Listeria monocytogenes and Australian Smallgoods: Detection and Control

By

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DECLARATION

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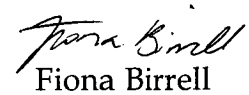

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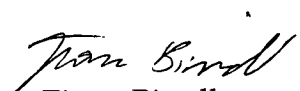

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Abstract

Despite its relatively low incidence, *Listeria monocytogenes* is an important food borne pathogen due to the seriousness of symptoms in susceptible population groups. *Listeria* spp. are ubiquitous and are difficult to eradicate from the smallgoods environment. The pathogen is destroyed during heat treatment, however post-processing contamination can occur. Because *L. monocytogenes* can grow at 4°C on vacuum- and/or modified atmosphere packed (VP, MAP) smallgoods that typically have a shelf life of six to eight weeks, dangerous growth of the organism can occur in the absence of control measures. Several international listeriosis outbreaks involving smallgoods attest to this.

This thesis investigated the incidence of *Listeria* spp. in Australian smallgoods, and the influence of interspecies bacterial competition on growth of *L. monocytogenes*.

The first part of this study comprised a survey of 100 smallgoods products to investigate the incidence of *L. monocytogenes* in Australian processed meats. Although no endogenous *L. monocytogenes* strains were isolated during this study, other *Listeria* spp. were occasionally found. This encouraging finding suggests that the increasing application of HACCP and GMP by Australian smallgoods producers has decreased the incidence of *L. monocytogenes* in processed meats.

A substantial part of this thesis investigated the potential control of *L. monocytogenes* by competition with endogenous lactic acid bacteria (LAB). The study included exploring the basis of the "Jameson Effect", a phenomenon that occurs when bacteria are grown in mixed cultures and inhibition of growth of all species is observed when one species reaches stationary phase, (i.e. 'maximum population density', MPD, or 'maximum carrying capacity', MCC of the environment). The "Jameson Effect" may be caused by a number of factors including; for example, bacteriocins, organic acids and nutrient depletion. This study explores the role of non-specific factors (i.e. pH and nutrient depletion) in the "Jameson Effect" and, by inference, the importance of species- specific interactions.

Endogenous LAB for use in competition studies were isolated from commercial sliced ham and identified. These strains, *Lactobacillus sakei, Leuconostoc mesenteroides,* and *Leuconostoc carnosum,* are commonly found on VP/MAP processed meats.

Another substantial aspect of the thesis was to determine whether nutrient depletion, pH or excreted soluble metabolites could explain the "Jameson Effect" for inhibition of *L. monocytogenes* (Scott A). The results of the study suggests that competition among bacteria for nutrients is a major factor leading to the "Jameson Effect" and that there is little need to invoke production of species- specific inhibitors to explain the effect.

Initial experiments to investigate the nature of the "Jameson Effect" were conducted in simple broth systems. These studies found that low pH and the presence of bacterial metabolites in the broth medium did not reduce MPD in the presence of adequate nutrients. This suggested that the "Jameson Effect" observed in broth systems could simply be symptomatic of nutrient depletion.

In support of this conclusion, consistent results were obtained in MAP ham studies undertaken to validate results of the broth studies under realistic commercial conditions. Thus, it was concluded that the role of lactic acid bacteria (which dominate VP/MAP processed meats) should not be underestimated when attempting to understand and control the risk of *L. monocytogenes* in refrigerated, VP/MAP processed meats.

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CONTENTS

ABBREVIATIONS

a_w	Water activity
CAMP	Christie, Atkins, and Munch-Peterson
E. coli	*Escherichia coli*
F_o	Control-freshly prepared media
GMP	Good Management Practice
HACCP	Hazard Analysis Critical Control Points
L. denitrificans	*Listeria denitrificans*
L. innocua	*Listeria innocua*
L. ivanovii	*Listeria ivanovii*
L. monocytogenes	*Listeria monocytogenes*
L. welshimeri	*Listeria welshimeri*
LAB	Lactic acid bacteria
Lb. plantarum	*Lactobacillus plantarum*
Lb. sakei	*Lactobacillus sakei*
Lc. carnosum	*Leuconostoc carnosum*
Le. mesenteroides	*Leuconostoc mesenteroides*
MAP	Modified Atmospheric Packaging
MRS	De Man, Rogosa Sharpe Broth
P. fluorescens	*Pseudomonas fluorescens*
pK_a	Dissociation constant for acid
RTE	Ready-to-eat
R. equi	*Rhodococcus equi*
S. aureus	*Staphylococcus faecalis*
S_o(pH_)	Unmodified 'spent' broth (pH and nutrient not altered)
S_o(pH7)	pH adjusted to 7, no dehydrated media added
S_o+N(pH_)	Dehydrated media added, no pH adjustment
S_o+N(pH7)	pH adjusted to 7 and dehydrated media was added
S_o+N(pH-S)	Dehydrated media added, no pH adjustment
Spp.	Species
$T_{equivalent}$	Temperature beyond which one bacterium grows faster than another bacterium.
T_{min}	Notational minimum temperature for growth
TSB-Ye	Tryptone Soya Broth with 0.6% Yeast
VP	Vacuum Packed

1 *Listeria monocytogenes* and Australian Smallgoods: Detection and Control

1.1 Introduction

The food borne pathogen *Listeria monocytogenes* has been studied extensively all over the world, although predominantly in modern Western nations, due to its ability to survive and grow in conditions traditionally accepted as safe measures for other food borne pathogens including low temperature (i.e. refrigeration), water activity (a_w) and pH. The foods associated with the cause of listeriosis in humans include Ready to Eat (RTE) foods, including modified atmospheric packed (MAP) smallgoods[1], due to the products relatively long shelf life and refrigerated storage conditions.

Listeriosis in Australia is mainly confined to the "high risk" groups including the young, old, pregnant and immunosuppressed populations, often described by the acronym "YOPI". Clinical symptoms of listeriosis can range from "flu like symptoms" to meningitis and septicaemia (Ryser and Marth, 1991). Recent findings have indicated that gastrointestinal tract symptoms can also result from listerial infection (Dalton *et al.*, 1997; Miettinen *et al.*, 1999; Aureli *et al.*, 2000).

All strains of *L. monocytogenes* are pathogenic, however, the level of virulence can vary between strains. Smith *et al.* (2003) conducted a dose response trial with Rhesus monkeys. In that study monkeys were injected with five different strains of *L. monocytogenes* isolated from human food borne outbreaks and monkeys. Of the ten monkeys infected with *L. monocytogenes*, half the births resulted in mortalities. The infective dose varied between strains for the five monkey foetal deaths. Three stillbirths were caused by a mix of 1/2a and 4b strains (monkey and human source respectively) of which the infective dose was around 10^8 CFU. A higher infective

[1] Smallgoods: goods sold in a delicatessen, especially meat such as sausage (Macquarie Dictionary, 1982).

dose of 10^{10} CFU was required for Scott A (4b). The lowest *L. monocytogenes* dose required to cause death of the monkey foetus, was by the strain 12443 (1/2a) that was around 10^6 CFU.

The incidence of listeriosis is relatively low which include 0.3 to 7.5 cases per million persons in Europe, 4.4 cases per million persons in the USA and 3 cases per million in Australia. The true incidence may not be known for these countries due to under-reporting. This is even more the case for developing countries (http://www.who.int/foodsafety/publications/micro/mra_listeria/en/).

The low incidence of listeriosis and low levels of *L. monocytogenes* found in RTE foods are highlighted in Ross *et al.* (2004) and Chen *et al.* (2003). Chen *et al.* (2003) used data from a food survey consisting of 31,705 food samples and listeriosis incidence data from the Northern California and Maryland Foodnet sites, to formulate dose response data. The Centre for Disease Control (CDC) reported 53 listeriosis cases during 2000 to 2001 in those sites. An exponential model was used in the study where the incidence was adjusted to 106 to allow for unreported cases based on Mead *et al.* (1999). The model suggested 67 of the 106 cases were caused by foods containing more than 100,000 CFU.g^{-1}. Only 2 of the 577 positive samples in Chen *et al.* (2003) survey of 31, 705 foods contained that amount of *L. monocytogenes* contamination. Ross *et al.* (2004) also reported the association of low numbers of cells ingested and the lower probability of acquiring listeriosis.

The frequency at which people are exposed to *L. monocytogenes* is much higher than the incidence of listeriosis which has lead to public health debate regarding the significance of ingesting low levels of pathogen in particular populations outside of the YOPI population (http://www.who.int/foodsafety/publications/micro/mra_listeria/en/).

This thesis is focused on the potential for control of *L. monocytogenes* on MAP meat by native competitive bacteria. Food Standards Australia and New Zealand (FSANZ) require that *L. monocytogenes* is not present in smallgoods that can support

its growth, based on a 25 g sample similar to approaches in some other nations. This criterion has become known as "zero tolerance". *L. monocytogenes* is ubiquitous in the environment, thus eradication is difficult and recall and litigation claims are costly to food manufacturers. A high dose is required before disease will occur for most consumers (Buchanan *et al.*, 1997; Smith *et al.*, 2003) but growth can occur in a range of RTE products, including smallgoods. If *L. monocytogenes* is already present on a product, control strategies to stop the pathogen from growing could be effective. The use of listeriostatic compounds, e.g. lactate and diacetate blends, may build a case for regulatory acceptance of these products as "not allowing the growth of *L. monocytogenes*" (Mbandi and Shelef, 2001; Glass *et al.*, 2002; Stekelenburg, 2003). This may result in removing the need for zero tolerance of *L. monocytogenes* in smallgoods. Another alternative involves "letting nature do the work" through the "Jameson Effect" (Jameson, 1962), i.e. inhibition of one bacteria by another, as observed during interactions of lactic acid bacteria (LAB) and *L. monocytogenes* (Coleman *et al.*, 2003).

The question arises "why is *L. monocytogenes* so important if the dose required to cause disease is so large?"

1.2 Microbiological Criteria for *L. monocytogenes* in Foods

The permitted level of *L. monocytogenes* in foods varies slightly between nations, however, a common rule stipulates zero tolerance. The Australian export trade is important to Australia's RTE industry, thus, compliance with zero presence of *L. monocytogenes* is necessary for acceptable export products. However, these standards were set when it was known that *L. monocytogenes* could cause death. More recently there has been an explosion of new information regarding control techniques for *L. monocytogenes* contamination and dose-response relationships and it is hoped this new information will bring to light new methods for controlling *L. monocytogenes* risk in foods without requiring zero tolerance.

1.2.1 Australia

FSANZ set the Australian Food Standards Codes (FSC) including acceptable levels of *L. monocytogenes* (Table 1.1). These standards were based on available documented information on *L. monocytogenes* ability to survive and grow on various foods (FSANZ, 2002). No *L. monocytogenes* is permitted to be detected in a 25 g sample of smallgoods.

Table 1.1. Criteria for *L. monocytogenes* in foods from the Australian Food Standards Code (FSANZ, 2002).

Food type	**Level of *L. monocytogenes*.**
Packaged cooked cured/salted meat and cooked crustacean.	Not detected in 25g
Soft cheese made from thermized milk changed to soft and semi-soft cheese	Not detected in 25g
Molluscs that have undergone processing other than depuration, packaged heat treated meat paste and pâtés.	Not detected in 25g
Ready-to-eat finfish.	100 CFU.g^{-1} (one sample in 5)
Unpasteurized milk and butter, where expressed permitted by States and Territories, raw milk cheese	Not detected in 25g

1.2.2 International

The United States of America, Food and Drug Administration (USFDA and USDA) also apply a zero tolerance level (not detected/25g) for *L. monocytogenes* in RTE foods (Smoot and Pierson, 1997). Denmark have adopted a similar strategy in some RTE foods, however, <10 CFU.g^{-1} is acceptable in heat treated and preserved foods that are not associated with growth of *L. monocytogenes*. In food where *L monocytogenes* does not grow, <100 CFU.g^{-1} is acceptable (Norrung *et al.*, 1999).

The European Commission (1999) also group RTE food into various categories according to the type of product and whether they support growth of *L. monocytogenes* (Table 1.2). Tolerable levels of contamination were derived from consideration of data collected from recorded food borne outbreak information and sporadic cases as levels > 100 CFU.g^{-1} were commonly found in implicated foods. On the other hand the regulatory policy on *L. monocytogenes* levels in RTE food in Canada is based on product and environmental testing along with food inspections

(Farber *et al.*, 1996). The foods are placed into three categories as shown in Table 1.3.

Table 1.2 The European Commission regulation of *L. monocytogenes* based on Farber *et al.* (1996).

Ready-to-eat food categories	**Level of *L. monocytogenes***
A. Foods heat-treated to a listeriocidal level in the final package	Not permitted in 25g
B. Heat-treated products that are handled after heat treatment. The products support growth of *L. monocytogenes* during the shelf life at the stipulated storage temperature.	Not permitted in 25g
C. Lightly preserved products, not heat-treated. The products support growth of *L. monocytogenes* during the shelf life at the stipulated storage temperature.	Not permitted in 25g
D. Heat-treated products that are handled after heat treatment. The products are stabilized against growth of *L. monocytogenes* during the shelf life at the stipulated storage temperature.	< 100 CFU.g^{-1}
E. Lightly preserved products, not heat-treated. The products are stabilized against growth of *L. monocytogenes* during the shelf life at the stipulated storage temperature.	< 100 CFU.g^{-1}
F. Raw, RTE foods.	< 100 CFU.g^{-1}

Table 1.3. Compliance criteria for *Listeria monocytogenes* in RTE (RTE) foods (Canada) (Farber *et al.* 1996).

Category	**Action level**	**GMP status**	**Immediate action**	**Follow-up action**
RTE foods causally linked to listeriosis (This list presently includes: soft cheese, liver pate, coleslaw mix, jelly pork tongue	>0 CFU/50g	n/a	Class I recall to retail level Consideration of public alert. Appropriate follow-up at plant level	
All other RTE foods supporting growth of *L. monocytogenes* with refrigerated shelf-life >10 days	>0 CFU/50g	n/a	Class II recall to retail level Consideration of public alert. Appropriate follow-up at plant level	
RTE foods supporting growth of *L. monocytogenes* with refrigerated shelf-life≤10 days and all RTE foods not supporting growth	≤100 CFU/50g	Adequate GMP	Allow sale	Appropriate follow-up at plant level
	≤100 CFU/50g	Inadequate or no GMP	Consideration of class II recall or stop sale	Appropriate follow-up at plant level
	> 100 CFU/50g	N/a	Class II recall stop sale	Appropriate follow-up at plant level

GMP - Good manufacturing practice.

1.2.3 Zero Tolerance: "An Outdated Map?"

The zero tolerance approach has been contested by many commentators (e.g. Smoot and Pierson, 1997; Chen *et al.*, 2003, Ross *et al.*, 2004). Smoot and Pierson (1997) state it is difficult, if not impossible, to completely eliminate *L. monocytogenes* from manufacturing equipment on a continuous basis. Smoot and Pierson (1997) concluded that improved cleaning practices in food manufacturing plants did not necessarily lead to reduced *L. monocytogenes* incidences. They suggested more emphasis should be focused on the consumer group, rather than the manufacturer, as only a limited section of the population is at risk to the pathogen. The following section discusses whether zero tolerance for *L. monocytogenes* is effective in protecting public health.

Guidelines suggested by the International Commission on Microbiological Specifications for Foods (ICMSF) outline recommended tolerance of *L. monocytogenes* (van Schothorst, 1994) (Table 1.4). ICMSF categorises microbiological hazards into 15 cases dependent upon two factors. The first is the severity of the hazard and the second is whether the hazard will be reduced, not changed, or increased during the normal conditions of handling between sampling and consumption. Food to be eaten by people within the YOPI group would be placed into cases 13, 14 and 15. The risk to the normal population is considered appropriate to sampling plans 10,11 and 12 where the risk is classified as "moderate, direct, potentially extensive spread". Addition of listeriostatic substances to smallgoods, such as lactate and diacetate salts (Mbandi and Shelef, 2001; Glass *et al.*, 2002; Stekelenburg, 2003), will keep *L. monocytogenes* levels low and could be used to build a case for regulatory acceptance of low levels of *L monocytogenes* in food containing such additives (*see* Section 1.5.3).

Table 1.4 The guidelines for the recommended tolerance of *L. monocytogenes* by the YOPI and non-YOPI population by categorising 15 hazards into groups (van Schothorst, 1994).

Intended consumer	Health hazard	Conditions in which food is expected to be handled and consumed after sampling in the usual course of events		
		Reduced degree of hazard	*Cause no change in hazard*	*May increase hazard*
Normal individuals	Moderate, direct, potentially extensive spread	Case 10 n=5 (≤100/g)	Case 11 n=10 (≤100/g)	Case 12 n=20 (≤100/g)
Highly susceptible individuals	Severe, direct	Case 13 n=15 (<1/375g)	Case 14 n=30 (<1/750g)	Case 15 (<1/1500g)

1.3 Dietary Exposure to *L. monocytogenes*

It was suggested in the previous sections that a high dose of *L. monocytogenes* is usually required before listeriosis results. Exposure to *L. monocytogenes* is determined by the prevalence and concentration of pathogen in foods, consumption of food per individual and control steps taken to prevent contamination of food. An extensive study was conducted in the USA in 2003 to determine the prevalence and the concentration of the pathogen in RTE foods (Chen *et al.*, 2003). The study of Chen *et al.* (2003), carried out by the National Food Processors Association, involved 31,705 food samples. Only 21 of the 577 positive samples contained levels of *L. monocytogenes* greater than 100 CFU.g^{-1}. *L. monocytogenes* was significantly more common in luncheon meats, delicatessen (herein referred to as "deli") salads and seafood salads packaged in stores compared to manufactured goods. However, 16 of the 21 samples containing > 100 CFU.g^{-1} *L. monocytogenes* were manufacturer-packed samples.

There is less research conducted in Australia on food borne listeriosis as compared to the USA study by Chen *et al* (2003). In comparison to the USA, Australia has a smaller population, thus, the annual number of exposures to an infective dose of *L. monocytogenes* is less, although it is similar on a *per capita* basis (Ross *et al.*, 2004).

1.3.1 Production and Consumption

There are no nationally compiled statistics of smallgoods production in Australia. Ross *et al.* (2004) provided estimates of Australia's consumption of RTE smallgoods. These estimates indicate annual production of 380-90,000 tonnes of processed meat. The average Australian consumes between 250-320g of RTE smallgoods per week (Ross *et al.*, 2004). The consumption is greater in the 30 to 60 age group for both sexes, however, men consume more smallgoods than woman. This higher proportion of consumption in males may be reflected in the higher number of male (28) to female (18) listeriosis cases as a result of the Pilgrim's Pride/Wampler Food listeriosis outbreak in the USA (Fox, 2004).

1.3.1.1 Listeriosis Cases Worldwide and Food Borne Outbreaks

The greatest proportion of food borne diseases in any Western nation is associated with *Campylobacter* and then *Salmonella* (CDI, 2004). *L. monocytogenes*, although not a common cause of food borne disease, receives much attention due to its high fatality rate, especially where neonates and foetuses are affected.

International listeriosis outbreaks were reviewed by Siegman-Igra *et al.* (2002). In Israel, Siegman-Igra *et al.* (2002) reported 161 listeriosis cases between 1995 and 1999. These cases were divided into two groups. Internationally, there were 1744 cases where 1250 (72%) were non-perinatal and 494 (28%) were perinatal. Mortality rates are also indicated below (Table 1.5) and vary between countries (Table 1.6). The death rate appears to be higher in non perinatal patients (Siegman-Igra *et al.*, 2002).

Table 1.5. Estimates of non-perinatal listeriosis internationally.

Country	UK[1]	USA, six areas[2]	Finland, Helsinki[3]	Spain, Barcelona[4]	Australia, Sydney[5]	Switzerland, Western part[6]	France[7]	Israel[8]
Study period	1967-1985	1986	1971-1989	1982-1999	1983-1992	1983-1997	1992	1995-1999
Total cases reported	722	246	74	31	84	122	225	156
Non perinatal	474 (66%)	179 (73%)	58 (78%)	29 (94%)	71 (85%)	57 (47%)	225 (100%)	87 (56%)
Mortality	34%	25%	20%	52%	32%	14%	24%	21%

(1) McLauchlin (1990); (2) Gellin (1991); (3) Skogberg (1992); (4) Nolla-Salas (1993); (5) Paul *et al.* (1994); (6) Bula (1995); (7) Goulet (1996); (8) Siegman-Igra (2002).

Table 1.6. Estimates of perinatal listeriosis internationally.

Country	UK[1]	USA, six areas[2]	USA, four areas[3]	Spain, Barcelona[4]	Australia, Melbourne[5]	Israel[8]
Study period	1967-1985	1986	1982-1999	1982-1999	1983-1992	1995-1999
Total cases	722	246	119	135	24	156
Prenatal	248 (34%)	67 (27%)	65 (55%)	21 (16%)	24 (100%)	69 (44%)
Postnatal death	19%	1%	11%	5%	8%	4%
Intra-uterine death	17%	21%	23%	14%	17%	41%
Total mortality	36%	22%	34%	19%	25%	45%

(1) McLauchlin (1990); (2) Gellin (1991); (3) Cherubin (1991); (4) Nolla-Salas (1993); (5) Craig (1996); (6) Siegman-Igra (2002).

1.3.2 Outbreaks Associated with Smallgoods

There are a variety of foods that may support the growth of *L. monocytogenes*, however, this thesis is concerned only with processed meats. Surveys of *L. monocytogenes* in various meat products have been undertaken in many nations. Australian outbreaks include *Listeria* contamination in pâté (Western Australia) and pre-diced chicken (South Australia) in1990 and 1996 respectively (Ross *et al., 2004*). The Western Australia outbreak reported 11 cases of which six were foetal/neonatal deaths and South Australia reported five cases with no deaths. International

outbreaks of a larger scale have occurred in countries with larger populations than Australia. For example, a multi-state outbreak of listeriosis in the United States in 2000 was associated with RTE turkey meat. The outbreak caused seven mortalities over a seven month period which included three miscarriages (Hurd *et al.*, 2001). A more recent outbreak in October 2002 in South Pennsylvania, USA, involved the consumption of RTE turkey and chicken from Pilgrim's Pride/Wampler Foods (CDC, 2002). Food sample strains matched factory environmental strains thus incriminating the food manufacturer. The outbreak caused eight deaths, three miscarriages and 46 illnesses (CDC, 2002).

1.3.3 Recalls

When *L. monocytogenes* levels in food fall outside the regulation levels, the manufacturer is required to recall all products associated with the suspect product sample. Acceptable limits differ among nations (*see* Section 1.2).

Food recalls result from recognition of food borne outbreaks or due to detection of *L. monocytogenes* in foods in routine sampling or environmental checks. Food borne outbreaks listed in Table 1.5 and 1.6 would have resulted in immediate recall of products associated with the outbreak once the source was recognised. Typically the identification of the source might take weeks to months. FSANZ, the Australian food standards regulator, advised six *L. monocytogenes* recalls in processed meats during the first half of 2004. The outbreak in USA due to Pilgrim's Pride/Wampler foods (CDC, 2002) resulted in a voluntary recall of 12 million kg of RTE turkey and chicken.

Control measures could be put in place to remove or prevent the growth of *L. monocytogenes*, thus, eliminating the need for the recalls of products with low levels of contamination. McLellan (2003) supported the use of irradiation techniques to prevent the need to dispose of all recalled products. This method would need to be accepted by consumers and regulatory bodies before it could be implemented. Also, as mentioned in Section 1.1, addition of listeriostatic compounds including salts of lactate and diacetate, could be utilised as a control measure to ensure that *L. monocytogenes*, if present, would not exceed the unsafe level of 100 $CFU.mL^{-1}$

proposed by ICMSF. The use of these compounds will be discussed in more detail in Section 1.5.3. A third control method involving "bio-control" will be considered below.

Other recalls are due to environmental testing. In a recent recall in Australia the smallgoods manufacturer recalled sliced beef, pork, lamb, silverside, ham, salami and chicken roll after an environmental sample showed contamination in the factory (Recall Search Facility, 2003). Recalls are expensive and can affect consumer confidence and may have contributed to the company going out of business.

In summary, the ideal regarding *L. monocytogenes* in foods would be "not present". This is not always possible, thus, the need for environmental testing within food manufacturing plants and on finished products is required, particularly for large food manufacturers (i.e. those who might expose many consumers). Under zero tolerance approaches, monitoring of smallgoods for the presence of *L. monocytogenes* is effectively discouraged, being both an additional cost to producers and requiring reworking or destroying all batches found positive despite that there is not a strong correlation between listeriosis and low doses. Control strategies to prevent the growth of *L. monocytogenes* may lessen the requirement to rework or recall products where contamination levels are low, as is usually the case (Buchanan *et al.*, 1997 and Ross *et al.* 2004).

1.3.4 Factors Affecting *L. monocytogenes* Growth

1.3.4.1 Characteristics

The genus *Listeria* are gram-positive, psychrophilic, and facultative anaerobic rods that belong to the *Clostridium* sub-branch together with *Staphylococcus, Streptococcus*, *Lactobacillus* and *Brochothrix* (Rocourt and Seeliger, 1985). There are seven species of *Listeria*, which include *Listeria ivanovii, L. monocytogenes, L. welshimeri, L. seeligeri, L. denitrificans, L. murrayi* and *L. grayi* (Rocourt, 1999; Volokhov *et al.*, 2002; Somer and Kashi, 2003). Only *L. monocytogenes* is considered pathogenic to humans (Ryser and Marth, 1991) although Perrin *et al.*

(2003) recently reported a fatal bacteraemia caused by *L. innocua* in a 62-year-old patient.

Any *Listeria* species detected on RTE meats is important information to food manufacturers because the growth conditions for most *Listeria* species are the same. If a non-pathogenic *Listeria* species was detected on a RTE meat sample, the environment may favour *L. monocytogenes* as well. This will be discussed further in Chapter 2.

1.3.4.2 Growth Factors Relating to the Smallgoods Industry

The growth potential of *L. monocytogenes* differs from other food borne pathogens. RTE vacuum packed (VP) and MAP meat products are of interest in this thesis due to the absence of a terminal heat treatment step before consumption of meats, the ability of *L. monocytogenes* to survive and grow at refrigeration temperatures and the long refrigeration shelf life of the products (Arizcun *et al.*, 1998; Aureli *et al.*, 2000). Thus, *L. monocytogenes* can grow in VP and MAP smallgoods products. Table 1.7 lists *L. monocytogenes* growth ranges and their relationship to meat smallgoods. The growth conditions have been reviewed by Lou and Yousef (1999). A brief description of the factors affecting *L. monocytogenes* growth on smallgoods follows.

Table 1.7 *Listeria monocytogenes* general growth range and growth characteristics suitable to Smallgoods (reproduced from Ross *et al.* 2004).

Environmental Factors	Lower Limit	Optimum conditions	Upper limit	Smallgoods
Temperature (°C)	-2 to + 4	30 to 37	~ 45	4.4 to 10.2 (summer) 2.3 to 10.4 (winter)
Water activity (a_w) (%NaCl*)	0.91 to 0.93 (12-14% NaCl)	0.970 (5% NaCl)	>0.997 (0.5% NaCl)	~0.970
pH (HCl as acidulant)	4.2 to 4.3	7	9.4-9.5	5.5 to 6.3
Lactic acid (water phase)	0 mM sodium lactate	Species dependent	800-1000mM, sodium lactate[1]	Product dependent

*Salt conversions (Chirife and Resnik, 1984)

[1] Tienungoon (1998)

1.3.5 General Growth Factors

1.3.5.1 Temperature

The temperature ranges for *L. monocytogenes* growth (see Table 1. 7) have been recorded from literature as early as Gray *et al.* (1966). Wilkins *et al.* (1972) later proposed the physiological temperature range for *L. monocytogenes* for maximum, optimum and minimum growth rate to be 45-50, 38 and 3°C respectively. More recent publications suggest that the minimum growth temperature for *L. monocytogenes* is as low as –1.5°C in VP roast beef however it was not able to grow in MAP at those temperatures (Hudson *et al.*, 1994). *L. monocytogenes* will not *grow* below –1.5 °C however it can *survive* in freezing temperatures as low as -18°C (Lou and Yousef, 1999).

The maximum temperature at which *L. monocytogenes* can survive and/or grow is of concern when foods require a heat treatment before consumption to ensure a safe product (i.e. milk and the step before production of RTE cooked meat products). Survival of *L. monocytogenes* at extreme temperatures is less of a problem for food manufacturers as most bacteria are destroyed when subjected to temperatures over 45°C. However, *L. monocytogenes* has been detected in High Temperature Short Time (HTST; 71.6°C for 15 seconds) pasteurised milk (Lou and Yousef, 1999).

1.3.5.2 Acidity (pH)

Bergey's Manual of Systemic Bacteriology (Sneath *et al.*, 1986) states that *L. monocytogenes* has a wide range of growth at pH values from 5.6 to 9.6 with optimal growth occurring at neutral to slightly alkaline pH values. Further studies have indicated *L. monocytogenes* can grow at lower pH depending on other factors such as temperature and water activity, with a minimum pH for growth of 4.3 reported (Conner *et al.*, 1986; Tienungoon *et al.*, 2000).

The physiological mechanisms involved in the ability of *L. monocytogenes* to tolerate and grow in acid conditions have received much attention in recent years. Genetic factors have been associated with sigma B and LisRK (Hill *et al.*, 2002).

Acid specific mechanisms include the glutamate decarboxylase (GAD) enzyme system. The system raises *L. monocytogenes* intracellular pH in an acid stressed environment. After uptake of glutamate by specific transporters, cytoplasmic decarboxylation of glutamate results in the uptake of an intracellular proton. The result is a net increase in extracellular pH due to the exchange of extracellular glutamate for the more alkaline GABA (γ-amino butyrate, reaction product). The higher the GAD activity in *L. monocytogenes* the greater the ability for the bacteria to survive in high acid environments. Another acid-specific mechanism to help bacteria to survive in low acid environments is the membrane bound F_1F_0-ATPase system that can export protons from the cell. This functions as a membrane channel of proton translocation.

1.3.5.3 Water Activity

The term a_w is used as an index of the osmotic potential of foods or laboratory media and is defined as the ratio of the vapour over a food or medium at equilibrium with the atmosphere above it to the vapour pressure of pure water at the same temperature (Troller and Christian, 1978). With regard to bacteria, this is the water available for growth. Knowledge of a_w is important for limiting *L. monocytogenes* growth. The optimum a_w for *L. monocytogenes* has been reported as 0.970 (Petran and Zottola, 1989), however, Ross (1993) and Tienungoon (1998) report the optimum a_w for growth of *L. monocytogenes* to be in the range 0.985 to 0.99. In that study the a_w on VP and MAP smallgoods products was on average 0.970 (*see* Chapter 2), therefore, such products can support *L. monocytogenes* growth. *L. monocytogenes* is not fast growing compared to *Escherichia coli* and *Clostridium perfringens*, however, it does have the advantage over other competing microbes in reduced a_w environments (Krist *et al.*, 1999). *L. monocytogenes* grows "comfortably" in RTE VP meats, however, it can also grow at lower a_w including high salt environments such as salted mushrooms (Junttila and Brander, 1989). The a_w and pH of the salted mushrooms were 0.954 (i.e. 7.5% NaCl conversion to water activity from Chirife and Resnik, 1984) and 5.9, respectively.

The salt tolerance of *L. monocytogenes* has been widely documented (Ross *et al.*, 2000). Sneath *et al.* (1986) states that *L. monocytogenes* can grow in Nutrient Broth supplemented with up to 10% (w/v) NaCl and other reports show growth at between 12 and 13% (Lou and Yousef, 1999). The morphology changes when *L. monocytogenes* is grown in food or media containing high salt levels. This was noted when *L. monocytogenes* was grown on a salt agar medium and colonies formed were star shaped (Lou and Yousef, 1999). Bacterial cells appear to elongate in the presence of salt (Brizin, 1975).

The salt tolerance mechanisms of *L. monocytogenes* include the ability to produce organic osmolytes (compatible solutes). These compatible solutes have no charge thus do not interfere with proteins and other cellular functions in the cell. They do, however, provide effective osmotic balance in the cell when the external environment presents a challenge including salinity, high temperature, freeze-thaw treatment and drying (Lippert and Galinski, 1992). The exogenous addition of proline (10mM), betaine (1mM) and carnitine (1mM) stimulated growth of *L. monocytogenes* under osmotic stress conditions at 37 and 10°C (Beumer *et al.*, 1994). Carnitine is found on animal products and betaine is found on plant products (Beumer *et al.*, 1994). Therefore, the presence of carnitine on animal products including meat, may contribute to *L. monocytogenes* ability to survive in low salt conditions. *oBA* that encodes proteins for a proline uptake is a generic factor associated with salt tolerance of *L. monocytogenes* (Hill *et al.*, 2002).

The survival of *L. monocytogenes* in low salt concentrations contributes to its survival in some salted smallgoods.

1.3.5.4 Biofilms

Another selective advantage of *L. monocytogenes* to survive in the food manufacturing environment is its ability to produce biofilms. Biofilms are defined as bacteria that exist in communities of microbial species embedded in a biopolymer matrix on some substratum (Zottola and Sasahara, 1994). The formation of a biofilm involves a multi-step process where the substratum first undergoes a conditioning

process that allows cells to be absorbed by weak reversible electrostatic forces (Montville, 1997).

Arizcun *et al.* (1998) examined various treatments to eradicate *L. monocytogenes* biofilms from food processing plant materials. The most effective was combination treatments using NaOH (100 mM, pH 10.5) and acetic acid (76.7 mM, pH 5.4) and heat application of 55°C for five minutes. Thus, a biofilm is hard to remove once formed on food manufacturing plant materials.

Biofilms make bacteria harder to remove and also protects them from disinfectants, thus, they are difficult to eradicate from the manufacturing environment.

1.3.5.5 Oxygen Requirements.

L. monocytogenes is generally considered to be a facultative anaerobic, mesophilic organism. Isolation of *L. monocytogenes* is generally easier in a partially oxygenated environment. *L. monocytogenes* grows in VP RTE meat products where the oxygen levels have been depleted (Mol *et al.*, 1971; Hansen, 1995; Miettinen *et al.*, 1999; Norrung *et al.*, 1999; Duffy *et al.*, 2000). Allende *et al.* (2002) observed no effect of *L. monocytogenes* growth when super-atmospheric oxygen (oxygen added to packs at greater than 70 Kpa) was added to salad packs.

1.4 Modes of Food Contamination

1.4.1 Contamination from the Environment

L. monocytogenes is widespread in the environment. This is of interest to the smallgoods manufacturing plant because of the increased risk of contamination via the environment outside the plant. Contamination could be brought into the factory via food process workers and/or raw material entering the premises. Contamination sources are discussed below.

1.4.1.1 Soil and Vegetation

L. monocytogenes can be found in dead and decaying vegetation and in soils. The species appear to survive for longer in moist soils and are more prevalent in early spring. The length of survival of the bacteria is associated with the type of soil. Environmental samples showed *L. monocytogenes* survival in moist soil (high a_w) to be 3-4 fold greater than non-moist soils (low a_w) (Fenlon, 1999).

1.4.1.2 Sewage

Considering *L. monocytogenes* is a pathogen to humans, it is not surprising to find the bacteria in human and animal waste. A report from England recorded a level of 700 to >18,000 CFU.L^{-1} in the primary tank of a sewage treatment plant (Watkins and Sleath, 1981). The drying effect (low a_w) of the sun seems to play a part in reduction of *L. monocytogenes* in sewage sludge (Fenlon, 1999).

The pathogen can also be found in the digestive tracts and faeces of domestic animals. Bailey *et al.* (2003) observed environmental sources of *L. monocytogenes* via domestic animals. The study disclosed a zero incidence of *L. monocytogenes* in Australian sheep and cattle however *L. ivanovii* was isolated from one dairy cow. This finding did not agree with other countries where *L. monocytogenes* was found in higher numbers (i.e. Hungary as high as 90%, Denmark 51%, Germany 33%, Yugoslavia 19%, Canada 14.5%, Scandinavia 3.1%). The high *Listeria* numbers in these countries were attributed to higher stocking rates and intensive farming.

Ham products were used in this thesis (Chapter 5), thus, determining the occurrence of *L. monocytogenes* in piggeries and pig faeces is important when understanding sources of contamination to smallgoods factories via the raw material entering the factory. A study by Beloeil *et al.* (2003) investigated the presence of *L. monocytogenes* in piggeries. The pigpens were tested for *L. monocytogenes,* where faecal matter, animal feed and soil may have been present. *L. monocytogenes* was found in 15% of samples.

1.4.1.3 Silage

Silage production requires the fodder to be fermented properly so that unwelcome bacteria such as *L. monocytogenes* can be inhibited. LAB predominate, creating a low pH that is unfavourable to *L. monocytogenes* growth. Good anaerobic fermentation by LAB can create a pH $\leq$ 4.5. When aerobic conditions occur in silage due to poor silage management, the growth of moulds are favoured and the pH of the silage can be increased, thus creating an environment to favour the growth of most bacteria. Poor-grade silage has been associated with animal and foetus abortion (Wesley, 1999).

In the smallgoods industry, animal feed may be an environmental source of *L. monocytogenes* in relation to contamination of raw materials. For example, if a piggery has an abattoir on the premises, cross contamination from feed sources may occur. Beloeil *et al.* (2003) also tested wet pig feed for the presence of *L. monocytogenes* and found 13% positive.

Listeria is nearly ubiquitous in the environments associated with raw materials and factory workers, thus contamination in food processing environments is difficult to eliminate.

1.4.2 Contamination by *L. monocytogenes* in Food Production Environments

It can be appreciated that *L. monocytogenes* is ubiquitous in the environment in contact with raw materials and factory workers. Additionally, the bacteria can grow and survive in the conditions of drains, floors, and equipment of food manufacturing plants. There have been many reports relating to contamination of *L. monocytogenes* in smallgoods manufacturing environments (Mbandi and Shelef, 2001; Amezquita and Brashears, 2002; Sim *et al.*, 2002; Suihko *et al.*, 2002).

1.4.2.1 Contamination on Food Production Lines

Even after thorough cleaning *L. monocytogenes* can re-establish itself on equipment over many years if good manufacturing practices (GMP) are not adhered to by food manufacturers (Dominguez *et al.*, 2001; Maijala *et al.*, 2001; Suihko *et al.*, 2002). The design of the smallgoods food manufacturing plant should include isolation areas for the packaging and slicing of the pasteurised meat product. This area should not be anywhere near raw material as stated by Mol (1971).

If GMP is not properly managed, raw material may come into contact with the slicer and/or finished product and cause contamination. The prevalence of *L. monocytogenes* in VP fish products was attributed to the fish coming into contact with raw product as contamination was occurring before retail (Dominguez *et al.*, 2001).

Re-contamination is also a problem on production lines and was the cause of a food borne outbreak in Finland traced to butter (Maijala *et al.*, 2001). After the outbreak, environmental samples were collected from the dairy plant and *L. monocytogenes* was isolated from two floor drains. The *L. monocytogenes* clone associated with the outbreak was identical to the clone discovered in the same dairy plant and butter sample in a similar environmental inspection in 1997. Suihko *et al* (2002) also reported the association of poor cleaning techniques with the prevalence of *L. monocytogenes* in food products. Ribotyping procedures were used to isolate *L. monocytogenes* from eight food manufacturing plants. Of the 362 food sample products analysed, 17% were *Listeria* species and 9% *L. monocytogenes*. The samples were taken after a two hour cleaning process that indicated a better cleaning process was required to ensure eradication of *L. monocytogenes*. From that study it was reported that the most common place to find *L. monocytogenes* was on conveyor belts (27% isolates after cleaning) and floors and drains (40% after cleaning). Failure to properly clean and disinfect food manufacturing machines led to contamination of poultry carcasses in a study by Miettinen *et al.* (2001). 62% of the raw broiler pieces, bought from retail stores, were positive for *L. monocytogenes* (Miettinen *et al.*, 2001).

L. monocytogenes contamination may sometimes be reduced or prevented by cleaning equipment and following GMP practices. However, these have not proved to completely eradicate the pathogen from the food manufacturing environment. Sometimes *L. monocytogenes* is able to adsorb and attach to food processing equipment and form biofilms as mentioned (section 1.5.5.4). Kalmokoff *et al* (2001), tested a number of *L. monocytogenes* strains for biofilm formation. Only one isolate (CLIP 23485) produced biofilms. The low incidence of *L. monocytogenes* forming biofilms was also studied by Sasahara and Zottola (1993) who suggested that *L. monocytogenes* is able to absorb onto equipment if primary colonizing bacteria are already established to form a biofilm.

1.4.2.2 Equipment Cleaning Problems

GMP includes efficient cleaning between production runs. Taormina and Beuchat (2002) studied the ability of *L. monocytogenes* to survive exposure to high pH cleaner solutions for 30 min at 4°C. Eight cleaning agents, chemical sanitizers and heat treatments were tested. The alkaline cleaning agents offered varying degrees of lethal action. The results indicated that efficient use of sanitizers (includes 50 or 100mg of benzalkonium chloride and cetylpyridinium chloride - common constituents of sanitisers) was required to remove *L. monocytogenes* from manufacturing equipment.

Some strains of *L. monocytogenes* are harder to eradicate due to their ability to attach to manufacturing equipment (Beresford *et al.*, 2001). *L. monocytogenes* binds well to a wide range of materials including the materials used for food manufacturing equipment. Beresford *et al.* (2001) reported the ability of *L. monocytogenes* to adhere to food processing materials, including rubber and stainless steel.

1.4.2.3 Hazard Analysis Critical Control Points (HACCP)

The Australian, USA and UK government agencies have encouraged the food industry to follow the Hazard Analysis Critical Control Point (HACCP), or similar, program (FSIS 2003, European Commission 1999 and Federal Register 2003). This

HACCP approach helps to consistently produce good quality and safe product if all steps are monitored and documented correctly. FSIS implemented the HACCP system to manufacturers of RTE meats and poultry products that may support the growth of *L. monocytogenes* (Federal Register , 2003). The final interim rule for control of *L. monocytogenes* included post-lethality treatment followed by antimicrobial treatment. The post-lethality treatment includes extra heating, pasteurisation or high pressure processing step. Antimicrobial treatment is defined as a treatment that suppresses or limits pathogen growth. Examples of antimicrobial compounds used today include diacetate and lactate salts or acids (Mbandi and Shelef, 2001; Glass *et al.*, 2002; Stekelenburg, 2003).

Labelling incentives are also included in the Federal Register (2003) that allows the manufacturer to include statements that may increase consumer confidence in the product. An example of the statements used may include "Sprayed with a solution of sodium lactate to prevent growth of *L. monocytogenes*". This sort of labelling could be directed at the YOPI population (see section 1.1).

From the above discussion it is clear that it is difficult to totally eradicate *L. monocytogenes* from production line equipment, therefore, limiting the growth of *L. monocytogenes* in the product may be a supplementary option. The best option would be to eradicate the pathogen from the source. The following section includes procedures used to reduce growth in already contaminated meat products.

1.5 Control Strategies

As stated in Section 1.4, *L. monocytogenes* is ubiquitous and hard to eradicate from food production environments. If the presence of the pathogen cannot be avoided, the next best strategy would be to keep the levels low by using a treatment that does not detract from the quality of the meat product.

1.5.1 Chemical Additives

Sodium polyphosphates and monoglycerides have been considered for their ability to inhibit *L. monocytogenes* on food products.

Sodium polyphosphate is a polymer of phosphoric acid. It is already used in the food industry as a food additive that is able to act as a buffer, emulsifier, dispersant, antioxidant, and sequestrant. It appeared to inhibit *L. monocytogenes* growth in the study of Zaika and Kim (1993). A hypothesis to explain this inhibition is related to the chelating mechanism of polyphosphates. The chelating agents may have removed essential metals via cation-binding sites on the cell walls of the pathogen. The growth of *L. monocytogenes* seemed to improve when metal cations (i.e. Mg^{2+}) were added to the medium.

Monoglycerides are naturally occurring emulsifiers that show antimicrobial activity against *L. monocytogenes*. The downfall of this potential antimicrobial is that a large concentration of the product is needed to inhibit the growth of *L. monocytogenes* and may change the sensory attributes of the product (Wang and Johnson, 1997).

Consumer preference is for RTE food products that look good, taste good, have a longer shelf life and are minimally processed. Lists of chemicals on labels may not be conducive to this image. Non-chemical control techniques may be more favourable to an increasingly health conscious society.

1.5.2 Natural Methods for Inhibiting *L. monocytogenes*

1.5.2.1 Honey

Taormina *et al.* (2001) studied the antimicrobial effects of different honey types on some food borne pathogens including *S. sonnei* (10305-98), *L monocytogenes* (V7) and *S. aureus* (ATTC 6538). Inhibition of *L. monocytogenes* was observed when bacteria were placed in catalase-treated buckwheat honey broth. The catalase was included to eliminate hydrogen peroxide activity, thought to be the inhibitory factor.

Inhibition was more common in *L. monocytogenes* without catalase added to honey, suggesting that peroxide activity is not the only antimicrobial agent. Taormina *et al.* (2001) reported that, in general, the most efficient inhibitory honeys were unprocessed honeys with a dark colour. Darker coloured honey was associated with greater levels of anti-oxidants. Heat treatment did not seem to alter the antimicrobial and antioxidant power of the honey. The species most inhibited by honey treatments was *S. aureus*.

1.5.2.2 Essential Oils and MAP

Tsigarida *et al.* (2000) reported the affects of MAP on *L. monocytogenes* growth. Other variables included oregano essential oil and other microflora including LAB and *Pseudomonas*. Their study was conducted on fresh meat. The samples with MAP film more permeable to oxygen, appeared to support the growth of *L. monocytogenes* while less permeable films supported growth of lactic acid, gram negative bacteria. In addition to this, the growth of *L. monocytogenes* was favoured on meat that was naturally contaminated with microflora. The most interesting finding from this study was that *L. monocytogenes* growth was less on sterile meat when compared to growth in oxygen permeable packages. Oxygen permeable packages enhanced the growth of *Pseudomonas* and, concurrently, stimulated the growth of *L. monocytogenes*. It was suggested that proteolysis caused by *Pseudomonas fragi* provided free amino acids for *L. monocytogenes*.

1.5.3 Organic Acid Salts

There is regulatory acceptance of lactate and diacetate salts as antimicrobials against *L. monocytogenes* in Australia and internationally. Formulations of salts of lactate and diacetate salts have been tested in various countries and their use as listeriostatic treatments and for shelf life extension has been approved. The Food Safety and Inspection Service (FSIS) published a direct final rule on March 2000, regarding the use of bacteriostatic compounds in meat products (FSIS 2003). The rule was stated in the Federal Register as "Food Additives for the use in Meat and Poultry Products: Sodium Acetate, Sodium Lactate and Potassium Lactate". The levels of compounds allowed in or on products were increased from 2 to 4% for lactates and 0.1 to 0.25%

for diacetate. The Office of Policy, Program Development and Evaluation (OPPDE) conducted a follow up interview after the ruling was made by FSIS, involving the results from nine establishments that used lactate and diacetate on their products. The points relevant in this interview included the "purpose" the ingredients were added to products for each of the nine establishments. Four stated they used the compounds for anti-microbial treatment while the others stated they used them to increase the shelf life. One establishment used lactate salt as a flavour enhancer. The level and ratio of lactate and diacetate used by establishments were broken down into two groups. Five used sodium lactate at levels up to 2% and the other four used a lactate/diacetate mix. This mix was made up of 1.4-2.86% potassium lactate and 0.1% of sodium diacetate (FSIS, 2003). The addition of a mixture of lactate and diacetate salts has been shown to increase the inhibition of *L. monocytogenes* on cooked bratwurst containing beef and pork (Mbandi and Shelef, 2001; Glass *et al.*, 2002) as compared to lactate salt alone. However, other authors report satisfactory inhibition with lactate salt alone (Stekelenburg and Kant-Muermans, 2001).

A further ruling for the use of additives on RTE food products issued by FSIS was implemented in October 2003. This final interim rule allows the application of antimicrobial agents but requires that products be labelled with information regarding listeriostatic compounds added to product. Antimicrobial agents can only be added if evidence exists to demonstrate their antimicrobial ability, e.g. to suppress *L. monocytogenes* growth.

The antimicrobial activities of the lactate and diacetate salts are not fully understood, however, some reports have suggested the following mechanisms of inhibition.

1.5.3.1 Water Activity

The first mechanism postulated is a_w reduction (Shelef, 1993; Stekelenburg, 2003). In standard meat manufacturing procedures, final product a_w is determined by how much NaCl is introduced to the meat. Sodium lactate at a level of 3.3% appeared to lower the a_w of processed ham products from 0.977 to 0.961 (Stekelenburg and Kant-Muermans, 2001). Stekelenburg (2003) reported a_w reduction on addition of lactate

salt (3%) to meat (i.e. 0.974 to 0.964). In that study *Lactobacillus sake* was inhibited, thus extending shelf life of the product. Because of the low minimum a_w that permits growth of *L. monocytogenes*, the a_w reducing effect of lactate is not expected to be the primary mechanism of inhibition on *L. monocytogenes* growth.

1.5.3.2 Undissociated Lactic Acid

The second mechanism is associated with the interaction of the pH of the RTE meat and the dissociation of organic acids or their salts. To obtain large ratios of undissociated to dissociated forms of lactic acid, the pH of the product should be near or below the pK_a of the organic acid. The pK_a is the log of K_a which is the equilibrium constant of dissociation of an acid in water (Eqns. 1.1 and 1.2). HA denotes the undissociated acid and A^- denotes the dissociated acid. The strength of the acid is indicated by the magnitude of K_a. For example, the larger the K_a value the stronger the acid (Chang, 1981) because it will dissociate more completely.

$$HA + H_2O \Leftrightarrow H_3O^+ + A^- \qquad \text{(Eqn. 1.1)}$$

$$K_a = [H_3O^+][A^-]/[HA] \qquad \text{(Eqn. 1.2)}$$

The pK_a of lactic acid at 25°C is reported to be 3.86 (Budavari, 1989).

Inhibition of *L. monocytogenes* by lactic acid is most strongly correlated with the undissociated acid concentrations. This is because this form of lactic acid is uncharged and lipophilic and, hence, can pass through the bacterial cell membrane. The intra-cellular pH is maintained close to neutral thus the equilibrium will favour dissociation and release of a hydrogen ion and acidification of the cell cytoplasm. Equation 1.3 can be used to calculate undissociated lactic acid concentration (Presser *et al.*, 1997).

$$[\text{Undissociated lactic acid}] = [\text{total lactic acid}]/(1 + 10^{pH-3.86a}) \qquad \text{(Eqn. 1.3)}$$

The more acid the product the greater undissociated product available. Concentrations of undissociated lactic acid in the range 5 – 10 mM were found by Tienungoon *et al.* (2000) to prevent *L. monocytogenes* growth. At pH typical of smallgoods (i.e. pH 6 - 6.5) a total lactic acid concentration of ~ 0.5 to 1 M would be

required to achieve this level of undissociated acid in the absence of other hurdles to *L. monocytogenes* growth.

1.5.4 Hurdles

L. monocytogenes is able to survive and/or grow at low temperature, a_w and pH (*see* Section 1.3.5). Multiple stresses, including low temperature, a_w and/or pH, can act synergistically to impose "hurdles" to bacterial growth. Each hurdle can require the cell to expend energy on physiological responses, eventually overwhelming the cell's ability to alter its physiology to survive yet another stressful environmental change. In other words, the term "hurdles" describes obstacles bacteria need to overcome to survive and grow in an environment (Hill *et al.*, 2002).

Not all hurdles can be applied in all foods and other protective processes are required. For example, long shelf life cook-chill foods, do not have intrinsic factors to prevent *Clostridium botulinum* from growing (Rodgers, 2003). These products are liquid or semi-liquid, with a_w greater than 0.970, suitable for *C. botulinum* growth (Gould, 1999). These products are marketed on their "fresh" appeal to be attractive to the food services market and, thus, cannot have a low a_w, chemicals added or strongly acid pH. An alternative would be to add a protective culture or to encourage the indigenous microbes naturally present on the food to compete with pathogens that might be present.

1.6 Microbiological Competition

When bacteria are grown in mixed cultures, inhibition may occur. The factors contributing to this can be species-specific and/or non-specific.

1.6.1 Suppression of Maximum Population Density

The growth of bacteria follows the well known sigmoid "bacterial growth curve" which includes *lag phase*, *exponential phase*, and *stationary phase* then death phase.

The maximum population density (MPD) is when bacteria reach the maximum level of viable cells. The MPD can be determined by observing the point at which the stationary phase is at the highest point. The cause of the stationary phase was stated to be related to the medium aging and not to intrinsic aging of bacteria (Causton, 1977; Ratkowsky *et al.*, 1983). It has been well documented that when most bacteria grow in a broth, they reach MPD of 10^8-10^9 CFU.mL^{-1} and stop growing. The reason for bacterial growth cessation is not fully understood, however, theories have been formulated which include bacteriocin production, toxic end products of metabolism, low pH and nutrient depletion. When bacteria are in co-culture, the MPD of one bacterium can also be suppressed by another bacterium. Often, specific chemical products such as bacteriocins or siderophores are involved, but another process not related to species specific inhibition has also been reported and has come to be known as the Jameson Effect (Stephens *et al.*, 1996). For the purposes of this thesis, mechanisms of inhibition other than bacteriocin and siderophore production will be described as non-specific factors.

1.6.2 Specific factors

1.6.2.1 Siderophores

Bacteria with siderophores, on the other hand, inhibit competitors by taking up the available iron in the environment surrounding bacteria. Iron is an essential element for growth of bacteria. In a study conducted to examine the competitive actions of 209 *Pseudomonas* species around 80% of inhibitory strains produced siderophores (Gram, 1993; Gram *et al.*, 2002). *Pseudomonas* inhibits the growth of *L. monocytogenes* (Gram *et al.*, 2002). However, *Pseudomonas* is the main spoilage organisms in many chilled food products, such as milk, chicken, meat and fish and, thus, are not good candidates as protective cultures for use on foods.

1.6.2.2 Bacteriocins

In this thesis, specific factors for microbial inhibition are considered to include bacteriocins, antibiotics and or siderophores. Bacteriocins are similar to antibiotics as both inhibit other bacteria, however, there is a critical difference between the two

inhibiting mechanisms. Bacteriocins have a narrow killing range, which is only active when the species to be inhibited is similar to the bacteriocin producing species (Riley and Wertz, 2002).

Some bacteriocins have a wider range of inhibitory action compared to others. Additionally, the environmental factors in the food medium appear to have an effect on production of some bacteriocins. Duffes *et al.* (1999b) reported that low temperature and neutral to weak acidity favoured *Carnobacterium piscicola* V1 bacteriocin production in a cold smoked salmon system stored at 4°C. *L. monocytogenes* was reduced to less than 10 $CFU.g^{-1}$ on the salmon after 28 days. On the contrary, Wessels and Huss (1996) found that *Lactococcus lactis* was not able to survive and produce bacteriocin on lightly preserved fish with similar unfavourable conditions. Bacteriocins produced by intestinal *Lactobacillus* isolates, were found to be inhibitory to a larger range of microorganisms compared to those from *Lactobacillus delbrueckii* (UO004) (Boris *et al.*, 2001). Low incidence of *L. monocytogenes* in cheese in Morocco was noted and studied by Benkerroum *et al.* (2000). Moroccan fresh cheese, jben, uses natural starter cultures including *Lactococcus lactis* that may produce bacteriocins. Benkerroum *et al.* (2000) attempted to isolate bacteriocin producing strains and to assess their effectiveness in the control of *L. monocytogenes* in jben. Inhibition of *L. monocytogenes* was found to be caused by a bacteriocin produced by *Lactococcus lactis* (CCMM/IAV/BK2) together with other fermentation end products produced during cheese manufacture. Rodriguez (2001) investigated three bacteriocin-producing LAB strains isolated from milk during the manufacture and ripening of a semi-hard cheese manufactured from raw milk. *L. monocytogenes* appeared to have a higher chance of survival in cheeses made with non-bacteriocin producing starter cultures.

The actions of purified bacteriocin against *L. monocytogenes* growth was studied on smoked fish for the first time by Duffes *et al.* (1999b). The bacteriocin from *C. piscicola* V1 was found to have the most inhibitory action against L. *monocytogenes* growth.

Bacteriocin producing strains of bacteria can be used by the food industry if the bacterium does not cause spoilage or alter the organoleptic qualities of the product.

1.6.2.3 Bacteriocin Producing Bacteria not Suitable for Addition to Food

Bacteria not suitable for bio-preservatives on a commercial scale have also been identified in the literature. Egan *et al.* (1980) compared the ability of *Microbacterium thermosphactum* (now known as *Brochothrix thermosphacta*) and *Lactobacilli* to spoil sliced luncheon meats. *Lactobacilli* bacteria are common inhabitants of cooked meat surfaces and *Microbacterium thermosphactum* is also present on cooked meat surfaces, however, it is not as common. A taste panel assessed the aroma, off flavour and acceptability. *M. thermosphactum* was found to score low in all three of these qualities and caused spoilage when levels reached $\sim 10^8.g^{-1}$. Homofermentative LAB did not induce evidence of spoilage until after 21 days, and some time after the population level reached $10^8.g^{-1}$. Heterofermentative organisms led to spoilage defects earlier than homofermentative strains.

While many reports have shown evidence that bacteriocins inhibit *L. monocytogenes* in VP RTE foods, that this is the only, or the dominant, mechanism remains equivocal (Kalmokoff *et al.*, 1999; Leroy and de Vuyst, 2001; Amezquita and Brashears, 2002; Jacobsen *et al.*, 2003). Limiting factors, as discussed by Duffes *et al.* (1999a), include the re-growth of *L. monocytogenes* after bacteriocin action ceases and the effect of an imbalance of micro-biota which may favour spoilage organisms.

1.6.3 Non-Specific Factors: Jameson Effect

The "Jameson Effect" describes the phenomenon that, when grown in co-culture, when one bacterium reaches MPD it causes the other to enter stationary phase at the same time, irrespective of its concentration. This hypothesis can be described by Figure 1.1. "A" is one bacterium and "B" another. When the population of "A" reaches MPD, "B" stops growing and goes into stationary phase "early", i.e. at a cell

density lower than normally associated with onset of stationary phase in that environment.

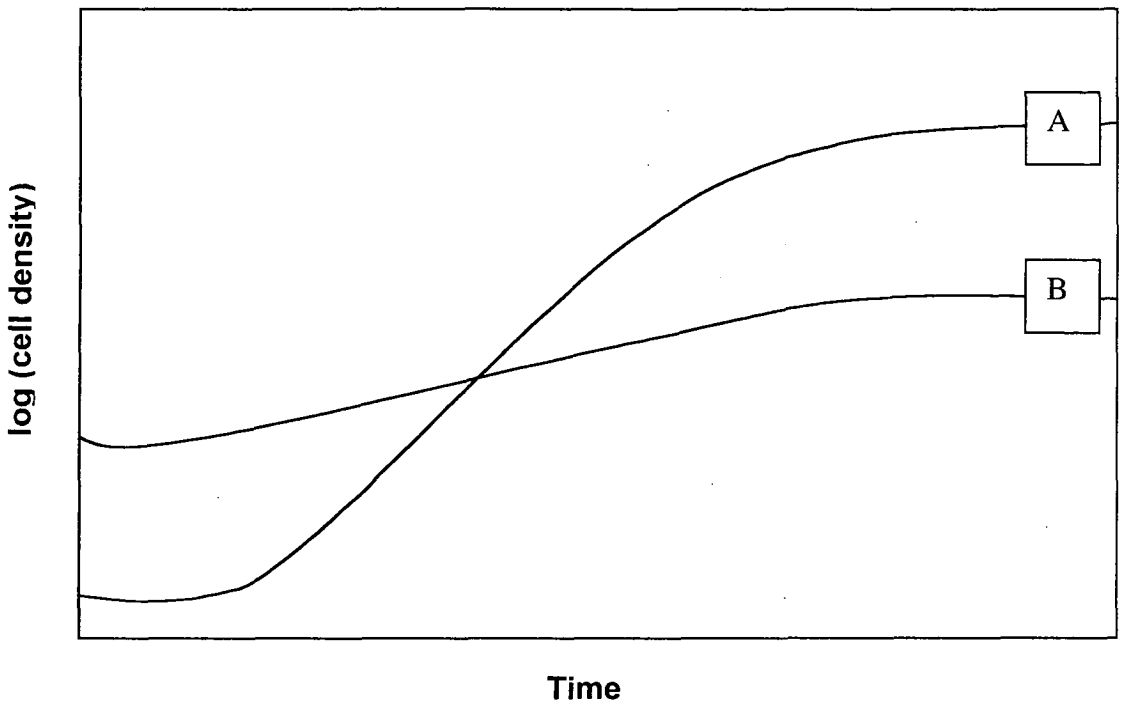


Figure 1.1 The growth of bacteria in co-culture. Despite initially being present in lower numbers, A is the first bacteria to reach MPD. B is suppressed by A reaching MPD and enters also stationary phase.

This suppression of growth in co-culture has been observed by various researchers in both broths and foods (Jameson, 1962; Coleman *et al.*, 2003). The role of *C. piscicola* in the Jameson Effect on cold smoked salmon has received considerable attention. *C. piscicola* make up a substantial part of the natural spoilage biota on cold smoked salmon but are less common on VP meat products (Hansen, 1995) and may be the reason that only relatively low levels of *L. monocytogenes* are found on cold-smoked salmon. Non-bacteriocinogenic producing strains of *C. piscicola* have been shown to inhibit *L. monocytogenes* in interaction studies (Buchanan and Bagi, 1997; Duffes *et al.*, 1999b; Nilsson *et al.*, 1999).

The Jameson Effect has also been reported on RTE meat products (Buchanan and Bagi, 1999; Duffes *et al.*, 1999b; Nilsson *et al.*, 1999). Grau and Vanderlinde (1992) reported a high incidence of *Listeria* on VP RTE corned-beef. At 0.1°C storage

L. monocytogenes growth stopped when all other bacteria reached 10^8 CFU.g^{-1}. Studies with cooked ham products showed that *L. monocytogenes* entered stationary phase when a non-bacteriocinogenic strain of *L. sake* reached 10^8CFU.g^{-1} (Stekelenburg and Kant-Muermans, 2001). In that trial, MPD for *L. monocytogenes* remained at a level between 10^4 to 10^5 CFU.g^{-1} once *L. sake* reached 10^8CFU.g^{-1}. Further studies involved a larger scale competition trial which examined LAB competition on commercial RTE meat products (Amezquita and Brashears, 2002). The trial was carried out at 5°C simulating the likely retail storage of meat products. The bio-preservation system in that report relied on isolating bacteria that had fast growth rates in cold storage thus having the ability to "out-compete" *L. monocytogenes*. Non-spoiling bacteria were also a preferred bio-preservative. Two of the most inhibitory strains in Amezquita and Brashears (2002) study included *Lactobacillus casei* and *Lactobacillus paracasei*. The suggested cause of *L. monocytogenes* suppression was via nutrient depletion by the fast growing LAB.

Amezquita and Brashears (2002) used the 16S r DNA sequence analysis method for identification of various LAB from RTE meat. The three most inhibitory strains against *L. monocytogenes* were identified as *Pediococcus acidilactici, Lactobacillus casei*, and *Lactobacillus paracasei*. Only *P. acidilactici* was proven to produce bacteriocins. The other two strains were suggested to be inhibitory due to organic acid production. Nilsson (1999) found strains of *C. piscicola,* that were unable to produce anti-listerial compounds did suppress the growth of *L. monocytogenes*. Buchanan and Bagi (1999) studied the mechanism of inhibition of *L. monocytogenes* by *P. fluorescens* (ATCC 33231), a non -bacteriocin producing strain of *Pseudomonas,* and concluded that environmental factors appeared to play an important role in suppression of *L. monocytogenes*. Because *P. fluorescens* cause spoilage, the authors suggested that *C. piscicola* would be more suitable than *P. fluorescens*. In summary, inhibition of *L. monocytogenes* without bacteriocin production is widely observed.

It may be concluded from the above that inhibition is not caused solely by bacteriocin production. Non-specific factors that contribute to inhibition of one

bacterium by another may include pH, nutrient depletion and metabolic end products.

1.6.3.1 pH Reduction

Acid environments inhibit the growth of *L. monocytogenes* and are completely inhibitory if the pH is below pH 4.3 (*see* Section 1.3.5.2). Breidt and Fleming (1998) observed competition between *Lactococcus lactis* and *L. monocytogenes*. *L. lactis* (LA221) is a non-bacteriocin producing strain. Inhibition appeared to be due to acid production by *L. lactis*, including production of lactic acid by both bacteria in co-culture.

1.6.3.2 Nutrient Depletion

L. monocytogenes requires certain substrates for growth including glucose and amino acids. The most important nutrient source for *L. monocytogenes* is glucose as the bacterium utilises a limited range of carbon sources (Premaratne *et al.*, 1991). Additionally, a dramatic drop in viability was observed in an experiment where the amino acid content of nutrient broth was limited (Herbert and Foster, 2001) because *L. monocytogenes* is an amino acid auxotroph, particularly at low temperatures. This may be of greater importance than glucose when determining the limiting nutrient for *L. monocytogenes* growth (Herbert and Foster, 2001), and is considered further in Chapter 3.

Buchanan *et al.* (1997) conducted an experiment with two strains of *C. piscicola*, (2762) a non-bacteriocin producing strain and (LK5) a bacteriocin producing strain. The inhibition of *L. monocytogenes* by the two strains seemed to be the same and a function of the growth rates of the two LAB. It was suggested that biotic factors such as peroxide production, pH depression or oxygen depletion were not associated with the inhibition. The timing of *L. monocytogenes* growth suppression generally coincided with *C. piscicola* entering early stationary phase growth where the limiting factor was nutrient depletion. It was suggested that the inhibitory effect could be

attributed to nutrient depletion. Bacteria growing in broth depleted of the nutrient inhibited slower growing bacteria.

On the other hand, adding nutrients to already depleted media, appeared to have no effect on growth (Nilsson *et al.*, 1999). This was also observed by Buchanan and Bagi (1999) who added amino acids, vitamins, minerals and carbohydrates to salmon. Neither *L. monocytogenes* nor *C. piscicola* (A10a) grew faster. Nilsson *et al.* (1999) study showed *C. piscicola* growing rapidly on cold smoked salmon at 5°C, however, no evidence supported that suppression of *L. monocytogenes* was due to bacteriocins or nutrient depletion.

1.6.4 End Products of Metabolism

Another reason for *L. monocytogenes* inhibition in mixed cultures may be due to toxic end products. Jason (1983) developed a model for monophasic growth of *E. coli* in batch cultures. He observed that MPD appeared to be governed by the accumulation of toxic end products rather than nutrient depletion. The model took into account a number of assumptions as stated by Jason (1983):

1. Substrate is freely available at all times and its concentration is never rate limiting.
2. The production and growth of each bacterium is accompanied by the generation of a constant mass of inhibitory substance (or substances)
    - specific growth rate declines linearly with growth in bacterial numbers per unit concentration of medium
    - initial generation time is unaffected by the concentration of medium or by inoculum numbers
    - the population density in the stationary phase is proportional to the concentration of the medium.

This situation is not the same as the batch growth of bacteria in foods, however, in which substrates must eventually become limiting and Jason's insights are included here for reasons of completeness.

1.7 Nutritional Requirements of *L. monocytogenes* and Lactic Acid Bacteria

As discussed above, competition for nutrients may cause suppression of one organism by another. In this section the nutritional requirements of *L. monocytogenes* and LAB that are typically present in high numbers on VP and MAP processed meats are explored.

1.7.1 Media for *L. monocytogenes* Growth

Brain heart infusion broth (BHI) is routinely used for *L. monocytogenes* growth (Verheul *et al.*, 1995; Buchanan *et al.*, 1997; Buchanan and Bagi, 1999; Cornu *et al.*, 2002). Tryptone soya broth and yeast (TSB-Y) has also been used for *L. monocytogenes* growth trials because the broth appears to improve recovery of heat injured cells (Knabel and Thielen, 1995) and increase the yield (essentially equivalent to MPD) of bacteria compared to other media (Huang *et al.*, 1993; Mellefont, 2000). TSB with 0.6% yeast extract (TSB-Ye) has been used for growth trials in unpublished studies on bacterial competition conducted in this laboratory (Davidson, *unpubl.*; Mellefont, *unpubl.*) that were pre-cursor to the current thesis. The ingredients for TSB and BHI are listed Appendix A38. It can be seen that TSB provides sufficient glucose for *L. monocytogenes* growth.

Welshimer (1963) formulated a recipe of nutrients required for *L. monocytogenes* growth which was updated by Premaratne *et al.* (1991). Both recipes are shown in Appendix A38. Premaratne *et al.* (1991) noted poor growth of *L. monocytogenes* in Welshimer broth. The addition of ferric citrate improved *L. monocytogenes* growth in Modified Welshimer Broth (MWB). However without the main energy substrate glucose, the medium was insufficient for growth.

The chemically defined medium (CDM) of Herbert and Foster (2001), is also listed in Appendix A38 for comparison. Herbert and Foster (2001) studied starvation mechanisms of *L. monocytogenes*. Along with MWB, TSB and CDM provide an adequate amino acid levels and glucose level for *L. monocytogenes* growth.

1.7.2 Carbohydrate Utilisation by *L. monocytogenes*

Glucose is the most preferred substrate for growth of most bacteria (Patnaik, 2000). However amino acids were reported as the first substrate utilised by *L. monocytogenes* (Herbert and Foster, 2001) and will be further discussed in this section below. Knowledge of the preferred substrate for growth of bacteria is important when bacteria are grown in a mixed culture. If bacteria have a similar preferred substrate, a "competition" for the main substrate may result in some bacteria being suppressed (Patnaik, 2000; Patnaik, 2001).

L. monocytogenes growth can be limited in the absence of certain sugars. Monosaccharides including glucose appeared to be the limiting substrate for growth (Pine *et al.*, 1989). Pine (1989) found *L. monocytogenes* species to have the faster growth from glucose compared to the other *Listeria* species. The bacteria appeared to grow less well in anaerobic conditions with the disaccharides lactose, sucrose and maltose as substrates.

Premaratne *et al.* (1991) showed a marked decrease in survival of *L. monocytogenes* (Scott A) after 10 days in treatments without glucose. Premaratne *et al.* (1991) found that *L. monocytogenes* (Scott A) required over 0.5% glucose for growth. This was also seen by Herbert and Foster (2001), who showed a fall in viable population of *L. monocytogenes* over the first two days of growth when glucose was absent from CDM. After two days the viability dropped considerably.

L. monocytogenes is unable to utilise the following carbohydrates; lactose (glucose and galactose), melibiose, sucrose, raffinose, sorbose, sorbitol, mannitol, galactose, galactose-1-phosphate, arabinose, ribose, xylose. In addition to this, *L. monocytogenes* is unable to utilise some organic acid substrates including acetate, pyruvate, lactate, citrate, succinate, alpha-ketoglutarate, malate and fumarate. This was first reported by Trivett and Meyer (1971), who studied the growth response of *L. monocytogenes* strains A4413 and 9037-7 to carbohydrates. It was noted that the *L. monocytogenes* strains tested did not grow with pyruvate and/or citrate cycle intermediates as the carbon and energy sources. Pyruvate is the hydrogen acceptor

for NADH to form lactic acid in the glycolytic pathway (Salminen and Wright, 1993). The absorption of pyruvate was pH dependant (i.e. absorption into resting cells at a pH of 5.0 which is the undissociated form of pyruvic acid), with *L. monocytogenes* able to absorb pyruvate in very acid products. The pyruvate oxidation system in cell-free extracts was most active at pH 7.0.

Many bacteria are able to utilise these substrates including *Pseudomonas fluorescens* (Barrett, 1953) and *E. coli* (Gray *et al.*, 1966). In studies by Farrag and Marth (1992) and Buchanan and Bagi (1999) increased growth of *L. monocytogenes* in the presence of *P. fluorescens* was attributed to *P. fluorescens* making amino acids available for *L. monocytogenes* growth by proteolysis.

1.7.2.1 Glucose Content of RTE meat

Minimally processed meats also require glucose for flavour (Bell and Gill 1983). Bell and Gill (1983) produced a cooked luncheon meat to examine the activity of amylase in relation to glucose production from starch. The glucose content of meat slurries was investigated and found to be released from pork starch. Less glucose was found in beef and mutton slurries. The glucose content was also examined on VP RTE sliced ham (Bautista *et al.*, 2000). The final ingredients of the meat consisted of 71.8% meat, 21.95% water, 3.5% sugar and 2% NaCl. D-glucose content was 0.01%. The glucose content was too low to add flavour to the meat therefore sugars were added. This was achieved by adding L-glucose and D-tagatose. These sugars were chosen as they were not utilised by a number of spoilage LAB and did not degrade the quality of the product. L-glucose has a sweet taste but is not utilised in the human body for ATP production. The above added sugars were not utilised by many bacteria on meat including *Staphylococcus aureus*, *L. innocua*, *Bacillus cereus*, *Salmonella Typhi*, *E. coli 0157* and *Yersinia enterocolitica* whereas *L. plantarum*, *L. acidophilus*, *L. brevis*, *P. damnosus*, and *L. mesenteroides* were able to utilise the sugars. Interestingly, *L. monocytogenes* and *L. mesenteroides* were able to utilise the sugars in anaerobic conditions. Thus, this control technique for spoilage would not be suitable when trying to control *L. monocytogenes* levels on VP sliced ham products.

1.7.2.2 *Amino Acids and Vitamins as Limiting Factors to* L. monocytogenes *Growth*

The amino acids in foods also need to be considered along with pH, temperature and a_w. Herbert and Foster (2001) found that *L. monocytogenes* showed a dramatic drop in viability when no amino acids were present in media. This may be of greater importance than glucose when determining the limiting nutrient for *L. monocytogenes* growth (Herbert and Foster, 2001). While many bacteria can utilize inorganic ammonium salts as a nitrogen source for synthesis of all 20 amino acids (Anraku, 1980), *L. monocytogenes* is an amino acid auxotroph. The essential amino acids it requires to be pre-formed in its environment include leucine, isoleucine, valine, methionine, arginine, cysteine and glutamine (Premaratne, 1991). *L. monocytogenes*' limited biosynthetic capacity results in the bacteria having a high nutritional requirement for certain amino acids. In addition to these amino acids, vitamins riboflavin, thiamine, biotin and thioctic acid are required for growth. Thus, nutrients required for growth of *L. monocytogenes* include sugar, vitamins, iron and several amino acids (Premaratne *et al.*, 1991; Beumer *et al.*, 1994).

Amino sugars can be used to substitute for glucose when they are glucosamine or N-acetylglucosamine (Premaratne *et al.*, 1991). Pine (1989) reported no growth of any *Listeria* species on media where lactose was the main substrate, however, the addition of N-acetylglucosamine and glucosamine stimulated the growth of *L. monocytogenes* strain 2382. This was also seen by Premaratne (1991), where cell wall digests from *Lactococcus lactis* produced N-acetylglucosamine and N-acetylnuramic acid, which appeared to break down the lactose, thus promoting *L. monocytogenes* growth. This may suggest *L. monocytogenes* has the ability to break down fungi and bacterial cell walls to aid in its survival on nutrient deficient media.

Beumer *et al.* (1994) observed the ability of the osmoprotectant compounds carnitine and betaine and amino acid proline to enable *L. monocytogenes* to withstand low a_w. High levels of carnitine are found in animal products including meat and milk. On the other hand, proline is found in high levels in plant products and very high levels are found in sugar beet. Beumer *et al.* (1994) reported significant growth of

L. monocytogenes when 10 mM proline, 1 mM betaine and 1 mM carnitine were added to broth with a low a_w. The ability of *L. monocytogenes* to utilise these osmoprotectants and compatible solutes for survival, may contribute to their presences in VP meats where carnitine is known to be present.

1.7.3 Lactic Acid Bacteria - Nutritional Requirements

LAB are commonly found among the natural micro-biota on VP or MAP RTE meats. Two common LAB found on RTE are *Leuconostoc mesenteroides* and *Lactobacillus sakei* (*see* Chapter 3).

Most LAB produce lactic acid as a metabolic end product of glucose metabolism. The general pathway for most homofermentative LAB is the break down of glucose via the glycolytic pathway. For every one mole of glucose broken down, two moles of lactic acid and two ATP are produced. *Leuconostoc* and group three of the *Lactobacilli*, do not always follow the glycolysis pathway to produce energy. The production of acetate can result when respiration is aerobic (Salminen and Wright, 1993).

LAB are able to utilise different sugars for growth. *L. mesenteroides* utilises ribose, arabinose, fructose, sucrose, tetrahalose, galactose and mannose. *L. sakei* utilises ribose, galactose, glucose, fructose, mannose, maltose, saccharose, trehalose and gluconate (Sneath *et al.*, 1986). These nutrients are similar to the nutrients desired by *L. monocytogenes*. The main substrate for growth is similar for all of these bacteria, thus competition for these nutrients is expected.

1.8 Aims of this Thesis

L. monocytogenes has been demonstrated as a causative agent of food borne outbreaks in various parts of the world (Gellin *et al.*, 1991; Bula *et al.*, 1995; Dalton *et al.*, 1997; Aureli *et al.*, 2000; Hurd *et al.*, 2001; Siegman-Igra *et al.*, 2002). The most recent, large food borne outbreak and recall of smallgoods products was in the

USA where 46 cases of Listeriosis and eight deaths were caused by contaminated RTE processed meat despite the recall of 27.4 million pounds of product.

There have been small *L. monocytogenes* outbreaks in Australia (*see* Appendix A37), including from processed meats, i.e. the risk to smallgoods consumers are documented (Ross *et al.*, 2004). Conversely, there have been recalls of products with a very low level of contamination that, at the end of their shelf life, apparently posed little risk to consumers. This was a result of the zero tolerance approach adopted in Australia.

The ability of LAB to suppress *L. monocytogenes*, has been well documented (Nilsson *et al.*, 1999; Katla *et al.*, 2001; Amezquita and Brashears, 2002; Mataragas *et al.*, 2002) and, if better understood, might be able to be exploited to enable some tolerance of *L. monocytogenes* in RTE foods.

The aims of this study involve investigation of the exposure of Australian consumers to *L. monocytogenes* in RTE processed meats including the frequency of contamination of product, levels of contamination and the potential for growth in the product. In particular, the potential of the normal contaminating microbiota of VP smallgoods to inhibit *L. monocytogenes* growth will be explored to support risk assessments models for *L. monocytogenes* in such products.

Specifically, a survey of smallgoods will be conducted over the two years of this study. The survey will be conducted in the summer months, as this is the time when lapses of temperature control are most likely to occur. The incidence of *L. monocytogenes* in smallgoods available for retail sale in Tasmania has not been studied since 1986 (Garland 1995).

Knowledge of the basic factors causing cessation of *L. monocytogenes* growth in a pure culture will be undertaken to form a base from which to understand what happens in co-culture. To determine the role of non-specific factors in the Jameson

Effect against *L. monocytogenes*, a range of bacterial species will be grown and cell-free spent broths prepared to mimic the environment the cells were in when they reached MPD. Possible factors causing suppression of *L. monocytogenes* will be examined using various non-bacteriocin producing bacteria such as *E. coli, P. fluorescens* and LAB isolated from RTE VP or MAP processed meat products.

Finally, if inhibition of *L. monocytogenes* by non-bacteriocinogenic cultures can be demonstrated in a model laboratory system, trials in commercial products will be undertaken to assess the reliability of the Jameson Effect and whether it can be manipulated.

2 Survey of *Listeria monocytogenes* in Australian smallgoods

2.1 Introduction: *Listeria monocytogenes* detection in smallgoods

2.1.1 Survey Objectives

Delicatessen (Deli), vacuum packed (VP) and Modified Atmospheric Packaged (MAP) meat samples were collected from a variety of retail outlets in southern Tasmania over several months to determine the incidence of *L. monocytogenes* in Australian smallgoods. Local manufacturers occupy a small portion of the retail market in Tasmania, therefore the survey encompassed product from a variety of Australian smallgoods manufacturers. Samples were collected during summer months when a higher *L. monocytogenes* prevalence might be expected due to warmer ambient temperatures (Tompkin, 2002). The survey was conducted primarily to assess changes in *L. monocytogenes* incidence compared to older studies in the published literature, and since the more widespread adoption of Good Manufacturing Practice (GMP) and HACCP by smallgoods manufacturers in Australia.

The Australian/New Zealand standard method (AS/NZS 1766.2.16.1:1998) utilised in this study is consistent with international standards for isolating *L. monocytogenes* in food products. This study assessed the sensitivity of the method for *L. monocytogenes* detection, reliability of selective media and the influence of competitive microbes. The influence of competitive microbes on VP/MAP meat was assessed using two methods. The first method involved spiking meat samples with different levels of *L. monocytogenes* to observe the influence of other meat microbes on *L. monocytogenes* detection level. The second method involved introducing a subculture step at 24 h in the secondary broth enrichment stage to assess the effect of possible overgrowth of *Listeria* spp. by naturally present microflora.

The efficacy of CHROM^{TM}agar Listeria selective plates (CHROM^{TM}agar Listeria LM850, CHROMagar Microbiology) was assessed in comparison to the selective plates used in the Australian/New Zealand Standard method. CHROM^{TM}agar

Listeria is used to more rapidly distinguish *L. innocua* positives from *L. monocytogenes* (Casolari *et al.*, 1994; Dominguez *et al.*, 2001). Resolution of *L. innocua* isolates from *L. monocytogenes* may take up to 10 days by AS/NZS 1766.2.16.1:1998. A purported advantage of using CHROM™agar Listeria is that it is less time consuming (i.e detection time is said to be 48 h for Part a and 8 days for Part b, *see* Figure 2.1) and eliminates *L. innocua* false positives. Limited published literature is available on Chromagar (Ottaviani *et al.*, 1997).

2.2 Materials and Methods

2.2.1 Sample collection

Deli and vacuum-packed meat samples were purchased during summer months from retail outlets in southern Tasmania. Samples were collected mid- and late-summer as the higher seasonal temperatures were considered more likely to permit microbial growth and to increase the probability of detection of *L. monocytogenes* on samples (Tompkin, 2002). 100 samples were taken from a mixture of national supermarket chains and smaller independent stores that sell fresh-sliced deli, VP and/or MAP processed meats (Table 2.1). Five samples of freshly sliced product were purchased to assess the cleanliness of the deli slicer in retail premises, as well as five samples each of four commercially produced VP/MAP processed meat products.

The pH and water activity of each sample were measured as follows. pH was measured on the meat surface and from meat macerated in an equal quantity of water (Orion pH meter 250A and electrode, Appendix 2). A portion of meat was macerated and the water activity determined with an Aqualab CX-2 dew point water activity meter (Appendix 2).

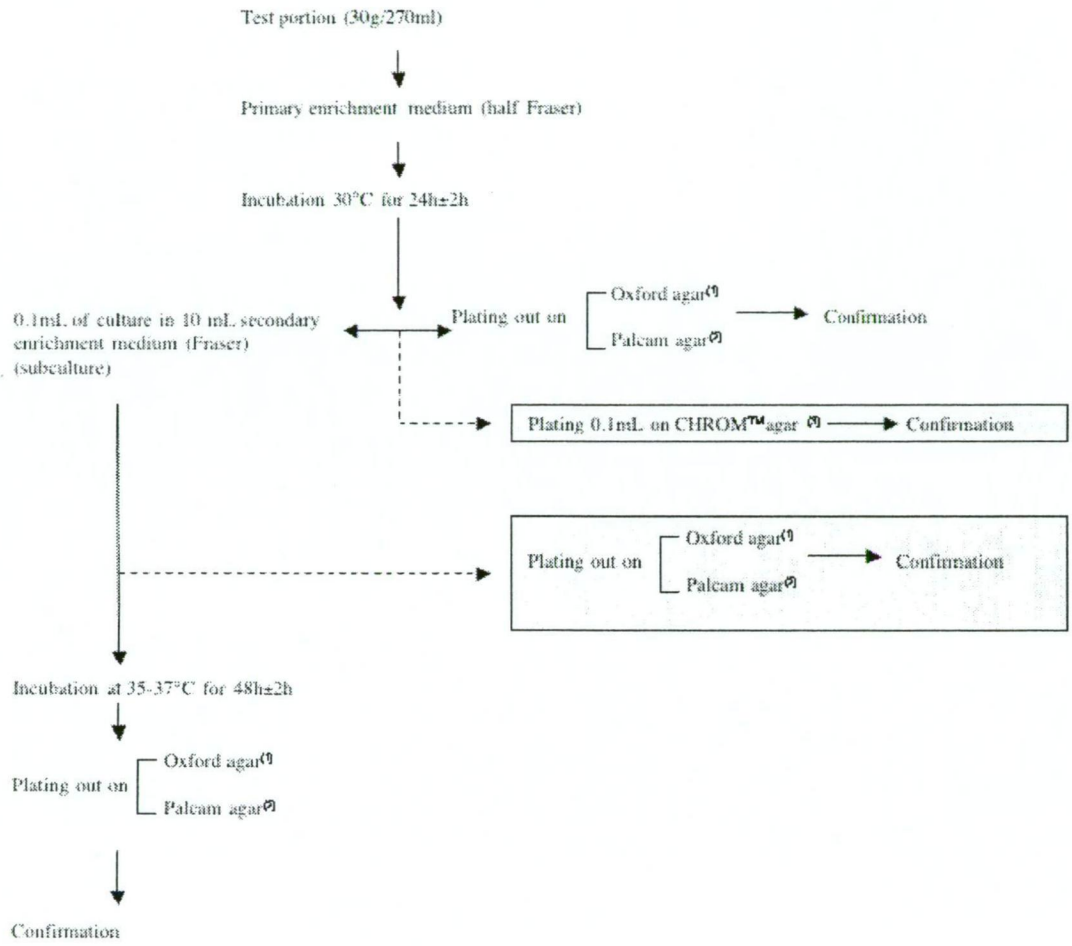
2.2.2 Sample Analysis

2.2.2.1 Method of detection : Australian/New Zealand Standard (AS/NZS 1766.2.16.1:1998)

The Australian/New Zealand Standard Method (AS/NZS 1766.2.16.1:1998) was used to isolate *Listeria* spp. and to identify *L. monocytogenes* (Figures 2.1a-c).

Table 2.1 Summary of samples collected for survey.

Lot Number	Deli meats	VP/MAP products
Lot 1: 25 samples	5 samples consisting of a variety of different deli meats from Supermarket 1: Each sample consisted of 100g.	4 VP/MAP sandwich meat products, consisting of five representative samples of each product. • shaved leg ham • prosciutto • paté cracked pepper • sliced honey ham
Lot 2: 25 samples	5 samples consisting of a variety of different deli meats from Supermarket 2: Each sample consisted of 100g.	4 VP/MAP sandwich meat products, consisting of five representative samples of each product. • breakfast bacon • paté • shaved baked English leg ham • unsmoked leg ham
Lot 3: 24 samples	4 samples consisting of a variety of different deli meats from small store 1: Each sample consisted of 100g.	4 VP/MAP sandwich meat products, consisting of five representative samples of each product. • leg ham, champagne • shaved Italian style mortadella • sliced ham • lean and tasty, 95% fat free ham
Lot 4: 25 samples	5 samples from small store 2: Each sample consisted of 100g.	4 VP/MAP sandwich meat products, consisting of five representative samples of each product. • pastrami • champagne ham • sandwich sliced mixed meats • kabanas



(1) Oxford agar plates are incubated for 24 to 48h at 35-37°C, aerobically
(2) PALCAM agar plates are incubated for 24 to 48h at 35-37°C, aerobically
(3) CHROM™agar are incubated for 24h only at 30°C, aerobically

Figure 2.1(a) Sample preparation and isolation of presumptive *Listeria* spp.

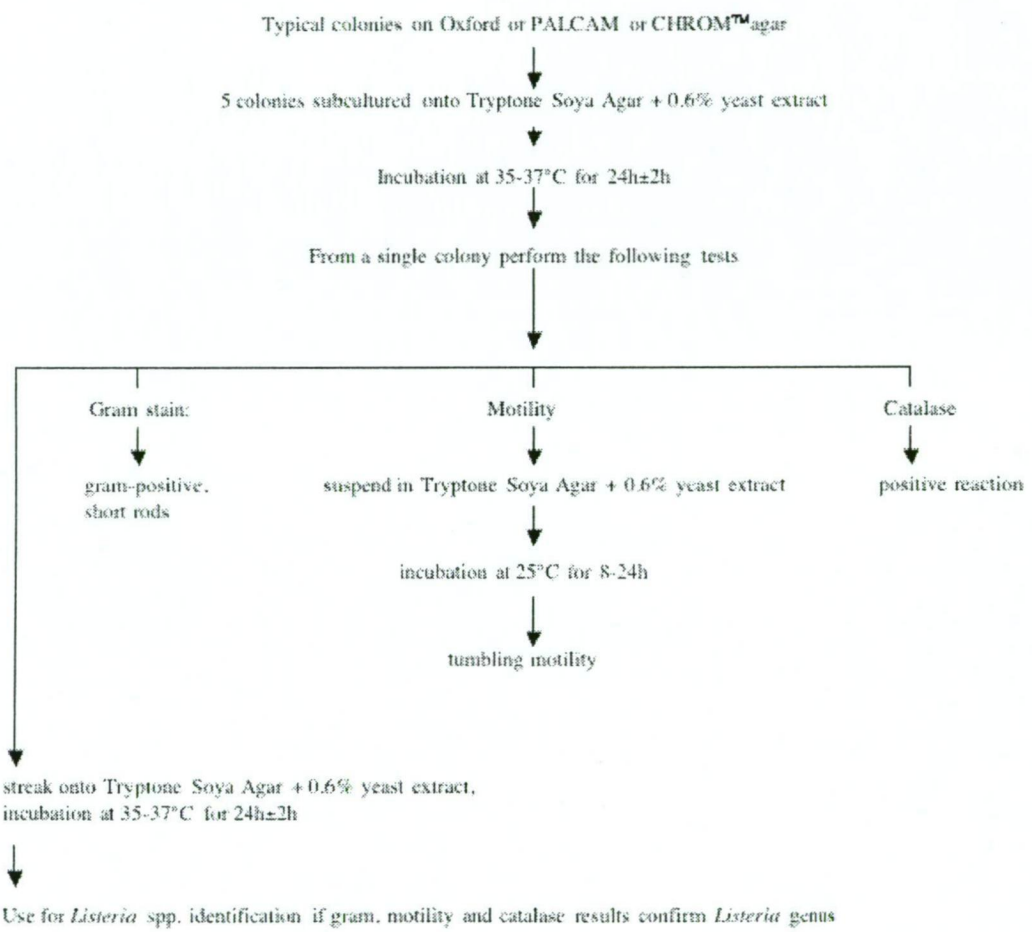
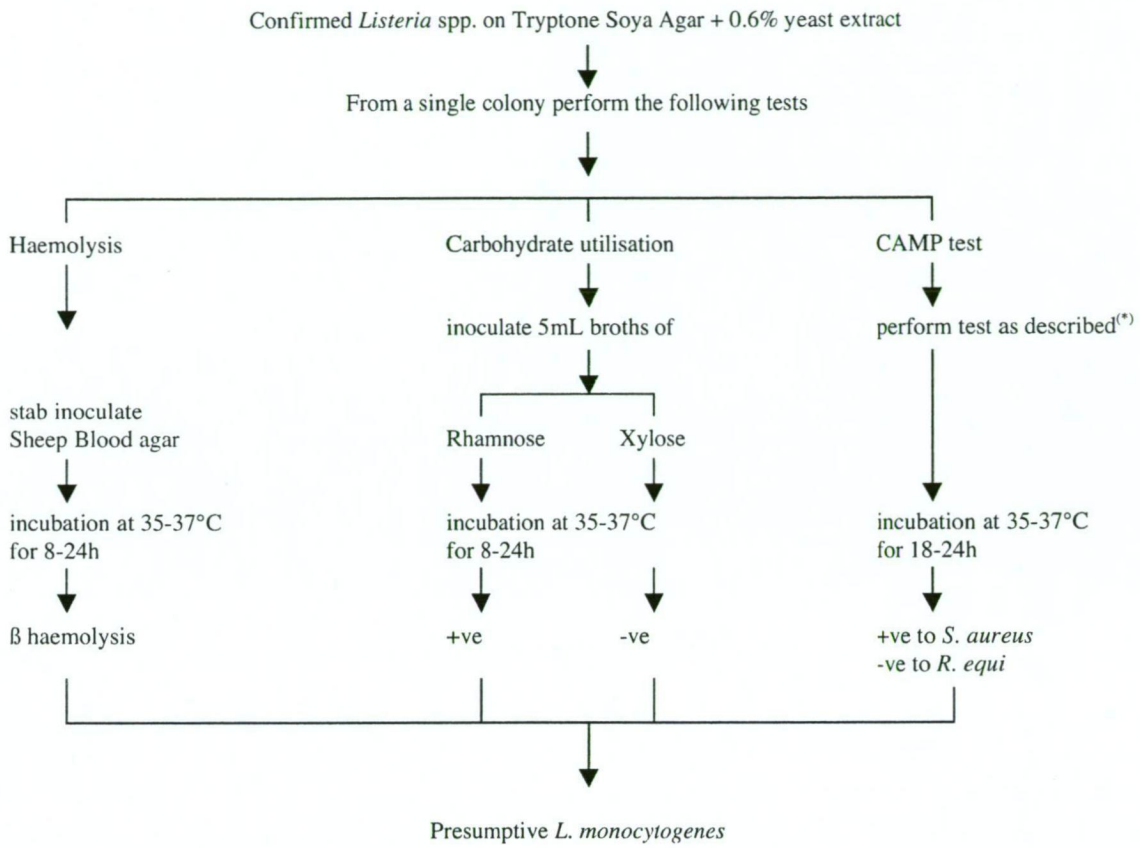


Figure 2.1 (b) Confirmation of *Listeria* spp.



*CAMP test-inoculation and interpretation

Inoculate thin Sheep Blood agar plates as per diagram below. Vertical lines represent streaks of *S. aureus* (S) and *R. equi* (R). Horizontal lines represent streaks of positive controls. Hatched areas indicate enhanced zones of haemolysis. The dotted line indicates the zone of influence of the *S. aureus* culture

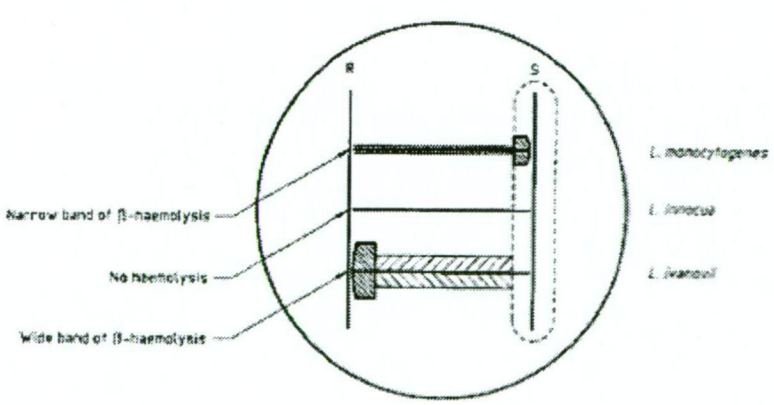


Figure 2.1 (c) Confirmation of *Listeria monocytogenes*.

The procedure for confirmation of *Listeria* spp. (Figure 2.1b) and *L. monocytogenes* (Figure 2.1c) are as follows:

- Presumptive positive colony taken from PALCAM or Oxford plate and 16 streaked onto a non-selective TSB-Ye plate then grown for 24 h at 37°C.
- Five colonies from each pure colony plate were re-plated onto five TSB-Ye plates and incubated for 24 h at 37°C. Each of the five pure colonies was gram stained, tested for catalase and motility. *Listeria* spp. are gram positive and positive for catalase and motility.
- Five colonies were streaked onto sheep blood agar plates to test for haemolysis.
- Five colonies were inoculated into carbohydrate solutions and tested for utilisation of L-Rhamnose and D-xylose. *L. monocytogenes* is the only *Listeria* spp. that utilises rhamnose but not xylose.
- Finally five colonies were streaked onto horse blood agar plates and tested against *Staphylococcus aureus* and *Rhodococcus equi* (i.e CAMP test). *L. monocytogenes*, *L. innocua* and *L. ivanovii* were streaked onto plates at right angles to *S. aureus* and *R. equi,* and compared to unknown *Listeria* spp. (see Figure 2.1c).

The flow charts in Figures 2.1(a-b) outline the procedures for isolation of *Listeria* spp., including variations to AS/NZS 1766.2.16.1:1998. Briefly, a 30g meat sample was aseptically transferred into a sterile stomacher bag containing 270mL of half Fraser broth (1/2FB) and homogenised in a Stomacher (Colworth 400, Appendix 2) for 2 minutes. 50mL of the stomached sample was retained and stored in Falcon tubes at 2°C for later enumeration of *L. monocytogenes* if a positive sample was found during the survey. Positive control samples were prepared for each sample lot of 25 by inoculating a colony of *L. monocytogenes* (Scott A) into 270mL Fraser broth and processing in the same manner as experimental samples.

2.2.2.2 Variations to the Australian/New Zealand Standard Method (AS/NZS 1766.2.16.1:1998).

The effect on the probability of recovery of *L. monocytogenes* following variation to the Australian New Zealand Standard Method was also explored. An additional subculture of the secondary enrichment broth was made 24 h after inoculation as

highlighted in Figure 2.1(a). A loopful of the secondary enrichment was plated onto Oxford and PALCAM agar plates. This additional subculture was included to assess whether false negative results could be caused by, for example, bacterial competition or the influence of changes to the composition of the media over time.

2.2.2.3 Sensitivity test for the Australian/New Zealand Standard method.

To assess the sensitivity of the AS/NZS 1766.2.16.1:1998 for detecting *L. monocytogenes*, random samples of VP/MAP products were 'spiked' with various levels of *L. monocytogenes* (Scott A). The samples were spiked at different levels to observe the influence of other meat microbes on *L. monocytogenes* detection. In addition to 'spiking' meat samples, positive controls were prepared by inoculating broths with *L. monocytogenes*. These 'spiked' broths with no added meat ('straight' broth) were processed in parallel with 'spiked' meat samples to assess *L. monocytogenes* detection levels without the influence of meat microbes or meat constituents.

The inoculum was prepared as follows. A loopful of *L. monocytogenes* (Scott A) from a well isolated colony on a 24h TSA plate was suspended in 10mL of TSB-Ye broth and incubated at 37°C for 24h to provide a stationary phase culture (1.9×10^9 CFU.mL^{-1}). The broth was diluted in 0.1% Peptone water and 0.85%NaCl, PW, as required.

For 'spiked' broth studies, 1mL of undiluted, 10^{-3} and 10^{-4} dilutions was added to 270mL FB to provide 3 broth samples containing levels of $7x10^6$, $7x10^3$ and $7X10^2$ CFU.mL^{-1} respectively (Table 2.3). Thereafter the procedure described previously in Section 2.2.2.1 was followed.

For 'spiked' product sample studies, VP/MAP products from Lots 3 and 4 were selected and 'spiked' as follows. From Lot 3 (see Table 2.1) champagne ham was selected arbitrarily for 'spiking'. As for broth samples, the ham was inoculated with levels of $7x10^6$, $7x10^3$ and 7×10^2 CFU.mL^{-1} (Table 2.2). The various levels of inoculum were added to the ham by pipetting 1mL of appropriate dilution into the stomacher bag containing the 30g meat sample and 270mL FB. The procedure described in Section 2.2.2.1 was then followed.

Table 2.2 "Inoculation levels for spiked broth and vacuum packaged meat samples"

Sample spiked	Inoculation level
MAP Champagne ham	~1 x 10^2
MAP Champagne ham	~1 x 10^3
MAP Champagne ham	~1 x 10^6
Inoculum into Fraser broth	~1 x 10^2
Inoculum into Fraser broth	~1 x 10^3
Inoculum into Fraser broth	~1 x 10^6
VP Pastrami	~1 x 10^2
VP Pastrami	~1 x 10^3
VP Pastrami	~1 x 10^4
VP Pastrami	~1 x 10^5
VP Pastrami	~1 x 10^6
Positive control :Inoculum into Fraser broth	One *L. monocytogenes* colony from TSB-Ye agar

From Lot 4 (see Table 2.1) pastrami was selected arbitrarily for 'spiking'. Pastrami samples were inoculated as described above with levels of ~10^2 10^3, 10^4, 10^5 and 10^6 CFU.mL^{-1} (Table 2.2). For each of the 5 'spiked' pastrami samples the procedure described in Section 2.2.2.1 was followed.

2.2.2.4 Typing of presumptive L. monocytogenes *isolates by PFGE*

Presumptive *L. monocytogenes* isolates were forwarded to the Institute of Medical and Veterinary Science (IMVS, Frome Road, Adelaide, SA) for strain typing by Pulsed Field Gel Electrophoresis (PFGE). Briefly, isolates were prepared for PFGE analysis by culture in BHI broth, pelleted and incorporated into an agar plug, lysed and digested. DNA was restricted with Sma enzyme, and the resultant fragments electrophoresed in CHEF DRIII tank for 22 h at 6V/cm by pulsed field with pulse intervals starting at 1 second and finishing at 20 seconds. The gel was stained with ethidium bromide and photographed under UV light. Finally the image was analysed with the assistance of GelCompar software version 4.11. (Wise, *pers. comm.*).

2.2.2.5 CHROMagar™ Listeria Method:

This newly introduced agar medium for differentiation of *L. monocytogenes* from *L. ivanovii* was tested in parallel with the Australian/New Zealand Standard method to assess whether it could more rapidly identify and eliminate false positives (*see* Figure 2.1a). Positive and negative controls for CHROMagar™ Listeria are

L. monocytogenes and *L. ivanovii* respectively. Positive controls were used to spike pastrami meat sample as described above. Briefly, 1mL of *L. monocytogenes* in TSB-Ye broth was inoculated into a stomacher bag with 30g of pastrami and 270mL of FB arbitrarily chosen from Lot 4 to the levels described in Table 2.2. These same inoculum levels were inoculated into straight half strength FB (i.e. no meat added). Negative and positive controls of *L. innocua* and *L. ivanovii* respectively were streaked onto CHROMagar™ Listeria plates.

2.3 Results

2.3.1 Detection of *Listeria* spp. and *Listeria monocytogenes*

Listeria spp. and *L. monocytogenes* detection results, as well as pH and a_w measurements, are presented in Tables 2 to 5 in Appendix 5. *L. monocytogenes* was detected in four of the 100 samples tested (Table 4, Appendix 5). However, after identification of these isolates by PFGE analysis, they were found to be identical to the control strain. It was concluded (see Discussion) that cross-contamination may have occurred between samples and the control. Thus, these "positives" are not considered further in the analysis of the survey.

L. monocytogenes was not detected in the remaining 96 samples surveyed, however, *Listeria* spp. were detected in eight samples and are detailed in Table 2.3. Of these eight samples, seven were found positive for *Listeria* spp by using the Australian/New Zealand Standard method-AS/NZS 1766.2.16.1:1998. *Listeria* spp. were isolated from the eighth sample (mortadella) using a variation to AS/NZS 1766.2.16.1:1998. The amended method detected six of the seven positive samples detected by AS/NZS 1766.2.16.1:1998 and one additional positive not detected by that method.

Table 2.3 Prevalence of *Listeria* spp. from a survey of 96 samples of processed meats sampled at retail.

Lot	Product		Method of detection		# of samples	*Listeria* spp. detected
	Source	Type	AN/NZ*	Modified**		
1	Product sliced at retail outlet	pancetta	+	+	1/1	*L. welshimeri* or *L. denitrificans*
		mortadella	–	+	1/1	*L. welshimeri* or *L. denitrificans*
	Vacuum-packed product	proscuitto	+	–	1/5	*L. welshimeri* or *L. denitrificans*
2	Product sliced at retail outlet	roast turkey	+	+	1/1	*L. innocua*
		roast pork	+	+	1/1	*L. innocua*
		honey ham	+	+	1/1	*L. innocua*
		silverside	+	+	1/1	*L. innocua*
		roast beef	+	+	1/1	*L. innocua*
Total of samples with *Listeria* spp.			7	8		

* Australian/New Zealand Standard method for detection of *Listeria monocytogenes*; AS/NZS 1766.2.16.1:1998

** The modified method involved an additional subculture of the secondary enrichment broth at 24h.

Two out of the four sample lots contained *Listeria* spp. Of these two sample lots, Lot 1 included contamination on slicer samples and process packed products. All five sliced samples were contaminated in Lot 2. Seven of these samples were recovered from product sliced in the store. Only one processor-packed product contained *Listeria* spp. (Table 2, Appendix 5).

2.3.2 Oxford *versus* PALCAM agar plates for detection of *L. monocytogenes.*

The efficacy of detection of *Listeria* spp. on the two *Listeria* selective media, Oxford and PALCAM agar, is shown in Table 2.4. For most samples, *Listeria* spp. were detected on both agar types. For one sample, mild-pancetta, *Listeria* spp. were detected on Oxford agar, but not on PALCAM agar. Conversely, for mortadella, *Listeria* spp. were detected on PALCAM agar, but not on Oxford agar. Both plates were needed to ensure detection of *Listeria* spp.

2.3.3 Modification of the Australian/New Zealand Standard method for detection of *L. monocytogenes*

The additional sampling at 24 h of the secondary enrichment broth yielded one positive sample for *Listeria* spp where the Australian/New Zealand Standard method did not (Table 2.3). The mortadella sample yielded a positive result in the secondary 24 h enrichment broth that was not detected on PALCAM or Oxford media at the 24 h primary stage or 48 h secondary stage.

Table 2.4 Detection of *Listeria* spp. on either PALCAM or Oxford agar after 24h primary enrichment and 24 or 48h secondary enrichment.

Product		Source	Recovery medium	
Number	Product		PALCAM	Oxford
1	Pancetta mild	24 h, primary	–	+
		24h, secondary	–	+
		48h, secondary	–	+
2	Prosciutto, san marino	24 h, primary	+	+
		24h, secondary	–	–
		48h, secondary	+	–
3	Mortadella	24h, primary	–	–
		24h, secondary	+	–
		48h, secondary	–	–
4	Roast turkey	24h, primary	+	+
		24h, secondary	+	+
		48h, secondary	+	+
5	Pork roast	24h, primary	+	+
		24h, secondary	+	+
		48h, secondary	+	+
6	Ham honey	24h, primary	+	+
		24h, secondary	+	+
		48h, secondary	+	+
7	Silver side	24h, primary	+	+
		24h, secondary	+	+
		48h, secondary	+	-
8	Roast beef	24h, primary	+	+
		24h, secondary	+	+
		48h, secondary	–	–
Total			17	17

2.3.4 Sensitivity of Australian/New Zealand Standard method for spiked samples.

All enrichments broths "spiked" with a level of $\geq 10^2$ CFU.mL^{-1} *L. monocytogenes* were positive after 24 h of incubation (in the primary enrichment) as evidenced by subculture on either agar media (see Table 2.5). For 'spiked' meat samples, a level of ~10^2 CFU.mL^{-1} *L. monocytogenes* was not detected on any media type. At levels $\geq 10^3$ CFU.mL^{-1} *Listeria* spp. were detected on all agar media.

Table 2.5 Detection of *L. monocytogenes* with different selective plates.

L. monocytogenes inoculum level CFU.mL^{-1} in 270ml	Straight Broth			Spiked samples		
	CHROM*	PALCAM	Oxford	CHROM*	PALCAM	Oxford
Lot 4-Pastrami						
10^2	+	+	+	-	-	-
10^3	+	+	+	+	+	+
10^4	+	+	+	+	+	+
10^5	+	+	+	+	+	+
10^6	+	+	+	+	+	+
Lot 3-Ham						
10^2	NT	+	+	NT	-	-
10^3	NT	+	+	NT	-	+
10^6	NT	+	+	NT	+	+
Control-one *L. monocytogenes* colony into FB broth	NT	+	+	NT	NT	NT

*where CHROM refers to CHROMagar™ Listeria
NT=Not tested

Pastrami samples (Lot 4) "spiked" with *L. monocytogenes* displayed similar detection levels on Oxford, PALCAM and CHROMagar™ Listeria agar after the primary 24 h enrichment. The quantity of *L. monocytogenes* on each plate was estimated by assuming each streak indicates an increasing number of bacteria. The greater the streak number the greater the bacteria present in the enrichment broth. The only notable difference between the three different selective media was with the sample spiked with a level of 10^3CFU.mL^{-1} of *L. monocytogenes* where CHROMagar™ Listeria agar displayed fewer colonies of *L. monocytogenes* compared to Oxford and PALCAM agar (Table 2.6).

Table 2.6 Positive *L. monocytogenes* colonies from spiked pastrami on Oxford, PALCAM and CHROM agar plates.

Inoculum level (CFU/270ml)	**Oxford (CFU/270ml)**	**PALCAM (CFU/270ml)**	**CHROMagar™ Listeria (CFU/270ml)**
10^2	19 (1st streak)	60 (1st streak)	8 (1st streak)
10^3	1(2nd streak)	18 (1st streak) 1 (2nd streak)	52 (1st streak)
10^5	15 (2nd streak)	22 (1st streak) 5 (2nd streak)	6 (2nd streak)
10^7	17 (3rd streak)	3 (3rd streak)	20 (2rd streak)
10^8	100 (3rd streak)	12 (3rd streak)	7 (3rd streak)

2.4 Discussion

No *L. monocytogenes* were detected in the 96 smallgoods/deli samples from Tasmanian retail outlets. However, some non-pathogenic *Listeria* spp. were found on deli meats (i.e. 5 out of 5 products from deli). From these results an insight into the efficacy of manufacturers' protocols for sanitising the food processing plant and equipment to prevent pathogen contamination may be inferred, and two points may be concluded from these results.

The first point relates to the effectiveness of smallgoods manufacturers at controlling the presence of *L. monocytogenes* on the product and in the factory environment. The survey resulted in zero detection of *L. monocytogenes*. This result is an improvement when compared to data from the Victorian State-wide Food Surveillance (statutory domestic) report 2000/2002 summarised in (Table 2.7).

Table 2.7 Incidence of *L. monocytogenes* in Australian smallgoods (Dunn *et al.*, 2000, 20001, 2002).

Smallgoods meat products	2000	2001	2002
Ham/sliced	9.4%	9.6%	11.6%
Manufactured meat/sliced	7.3%	7.5%	15%
Meat manufactured	5.7%	2.1%	1.2%

The absence of *L. monocytogenes* in this survey may suggest that Good Manufacturing Practices (GMP) and HACCP increasingly adopted by smallgoods manufacturers may have resulted in improvements in the level of control of *L. monocytogenes* in smallgoods. Many Australian food companies have adopted HACCP (NFS, 1998), including the smallgoods company who provided the ham for experiments in Chapter 5. Gombas *et al.* (2003) undertook a large survey of the incidence and concentration of *L. monocytogenes* in foods, including 9199 processed meats in the USA. They found similar results to this survey, reporting a prevalence of *L. monocytogenes* in luncheon meats of 0.89%, considerably lower than reported in earlier published studies.

L. monocytogenes contamination frequencies reported in this study appear to be low relative to recent Australian studies in the VP/MAP ready-to-eat retail section. The

Health Department of Western Australia (HDWA 1997) reported VP/MAP meats at retail to have a higher contamination rate than product at factory level of 13.4% and 4.3% respectively (Table 2.8). It should be noted, however, that the sample size of the survey reported here is relatively small (i.e. ~100 samples) when compared to other surveys cited (*see* below).

Table 2.8 Frequency (%) of contamination of Australian smallgoods products with *Listeria monocytogenes*, reproduced from Ross *et al.* (2004; Appendix 3a).

	Raw Product	Product at Factory	Product at Retail (remaining shelf life unknown)
Number of sample sets	2	7	28
Total number of samples	706	4032	1541
Mean (%) contamination rate from all surveys	12.9	4.3	13.4
Standard Deviation	9.3	2.4	15.5
Unweighted* mean	5.8	6.6	15.6

*The unweighted mean values shown in the above tables are calculated using the total number of positive samples among the total number of samples in the respective surveys.

This may be due to the smallgoods factory slicer and or packaging equipment contaminating finished cooked product as reported by Samelis *et al.* (1998). However, the observation that contamination frequencies at retail are higher suggests that contamination occurs subsequently or that growth to detectable levels occurs between production and retail. A study conducted in Denmark, (Fonnesbech Vogel *et al.*, 2001) reported contamination levels in the smoked salmon manufacturing process and found the slicer caused the most contamination. That study reported the detection of 63 *L. monocytogenes* types on the slicer, which was the highest source of contamination. Cleaning techniques were improved over six months, resulting in only six *L. monocytogenes* types found associated with the slicer and thus a reduction in the number of isolates detected. Requesting retailers to freshly slice five portions of ready-to-eat meat indirectly examined the cleaning efficiency of the delicatessen area in this survey. Slicing machines were suggested to be the greatest source of contamination in a Melbourne smallgoods factory (A. McCarthy, *pers. comm.*). The cleaning efficiency of slicing machines may have improved as evidenced by the non-detection of *L. monocytogenes* in VP/MAP products from that same manufacturer in this survey. To manage *L. monocytogenes,* the Melbourne processor instituted a HACCP programme. Control procedures at that factory

included controlled entry into unprocessed, slicing and packaging and finished product sections. Staff in different sections were assigned coded cloaks, hairnets and shoe protectors that shielded against any contamination from the raw meat room or outside environmental contaminants. Although GMP was enforced in this factory, contamination problems still occurred due to poor cleaning and disinfection of the floors. Stringent measures were used to keep the floor clean however problems arose due to the floor slowly drying thus creating an ideal environment for any surviving pathogens (McCarthy, *pers. comm.*).

As mentioned above, no *L. monocytogenes* spp. were detected from smallgoods in this survey, however, other non-pathogenic *Listeria* spp, were detected.
Only one VP/MAP sample contained *Listeria* spp. Other *Listeria* spp. were found in smallgoods sliced at the point of sale, which suggest a poor cleaning technique. Problems with contamination of *L. monocytogenes* on slicing machines were discussed above and in Section 1.4.2.1. *L. welshimeri* and *L. innocua* are both commonly found in food products (Table 2.9).

Table 2.9 Prevalence of *Listeria* spp. in various food products.

	Capita *et al.* (2001)	Casolari *et al.* (1994)	Luca *et al.* (1997)	Laciar *et al.* (1999)
Food type	Raw chicken	Raw meat and paté	Processed pork and cheese	Raw milk
Listeria spp.	95%	63%	85% (meat) and 17% (cheese)	3%
L. monocytogenes	32%	16%	25%	14%
L. innocua	66%	47%	31%	71%
L. welshimeri	7%	-	26%	14%
L. grayi	4%	-	15%	-
L. ivanovii	2%	-	0.5%	-

A recent study (Perrin *et al.*, 2003) indicates that the latter bacteria maybe be pathogenic in some circumstances. This leads on to the second point concluded from this survey, where the detection of *Listeria* spp. may indicate poor cleaning technique. Tompkin (2002) reported the relationship between detection of *Listeria* spp. and the likelihood of detecting *L. monocytogenes* among those *Listeria* spp. The likelihood of the presence of *L. monocytogenes* when *Listeria* spp. are detected seems to be related to the characteristics of each plant (i.e. unique ecology suited to *Listeria* strains). Tompkin (2002) suggested a response by processed meat

manufacturers to all positive *Listeria* spp. as though they were *L. monocytogenes*. As also mentioned in Section 1.3.5, all *Listeria* spp. grow under similar environmental conditions thus the presences of any *Listeria* spp. may involve delaying further production of product until the source of *Listeria* spp. is isolated.

Observations made using the Australian/New Zealand Standard method included the efficacy of selective *Listeria* agar plates at detecting *L. monocytogenes*. The selective plates PALCAM and Oxford agar appeared to show no significant difference in their ability to recover *L. monocytogenes* (Table 2.4). Other researchers have reported different results (Johansson, 1998; Cordano and Rocourt, 2001; Pinto *et al.*, 2001; Gracieux *et al.*, 2003; Kornacki *et al.*, 2003). Cordano and Rocourt (2001) conducted a survey of 2145 food samples including various food groups in Chile. The selective media used in their study included Oxford, LPM (Lithium chloride Phenylethanol agar) and PALCAM agar. They found PALCAM agar clearly better for isolating *L. monocytogenes* from shellfish. Variance in positive results from the same sample on different selective media emphasised the importance of using a variety of different selective media to eliminate false positives and negatives. The validity of the results obtained from using AS/NZS 1766.2.16.1:1998S are also brought into question by the results of Pinto *et al.* (2001) who reported the benefits of using *Listeria monocytogenes* blood agar (LMBA) together with PALCAM or Oxford agar. The LMBA reduced time and materials required for confirmation because at the end of the incubation period of the selective plates haemolytic zones were also observed. This reaction can be used to distinguish between the morphologically similar looking bacterium *L. innocua* which is non-haemolytic while *L. monocytogenes* is haemolytic. The selective agents in PALCAM and Oxford can prevent the growth of injured microorganisms Johansson (1998). This finding was also reported by Pinto *et al.* (2001). There has been debate about the efficacy of *Listeria* selective agar media. Bedie *et al.* (2001) carried out studies with anti-microbial compounds including sodium lactate, sodium acetate, sodium di-acetate. The study involved treating frankfurters with these compounds. The frankfurters were tested for viable *L. monocytogenes* using PALCAM and TSAYE agar. There was significantly more *L. monocytogenes* growth on TSAYE plates as compared to PALCAM plates. This suggests that bacterial cells were not all necessarily killed by the anti-microbial agents, but rather injured. Thus, the non-selective media allowed for recovery and colony formation of injured cells. This

observation is important because the selective media in question may inhibit isolation of injured cells that may otherwise repair themselves and become a potential risk in foods. This may indicate that a third non-selective medium, possibly CHROMagar™ Listeria agar, would increase the probability that the Australian/New Zealand Standard method would detect injured cells that are inhibited by the other selective media.

PALCAM and Oxford Agar plates are not sufficient to differentiate between *L. monocytogenes* from *L. innocua*. In this study CHROM™agar Listeria was used in parallel with the Australian/New Zealand Standard method and media to compare the efficacy of both methods for detecting *L. monocytogenes*. There is limited published information regarding the use of CHROM™agar Listeria to detect *L. monocytogenes* Coignard (2001).

No *Listeria* spp. were found in samples from Lot 4 thus no comparison was possible between the two approaches. However, conclusions can be drawn from the results of Lot 1 and 2 as *L. innocua* and other *Listeria* spp. were found after completion of the all tests by the Australian/New Zealand Standard method. If CHROM™agar Listeria had been included in these isolation procedures, time and money may have been saved due to elimination of false *L. monocytogenes* positives, including *L. innocua* and other *Listeria* spp. (See Tables 2 to 5, Appendix 5). This observation was supported by Sacchetti *et al.* (2003) who also compared a chromogenic agar medium with PALCAM and Oxford agar.

The cost differences between the three types of selective media used in the survey undertaken in this study were calculated (Table. 2.10).

Table 2.10 Cost of Australian/New Zealand standards and CHROMagar™ Listeria per 25 samples.

	Cost ($)	Comparative Cost ($)
Australian/New Zealand standards		
PALCAM base (7L)	171	50.00 (50-80 plates)
PALCAM supplement	80	32.00 (50-80 plates)
Listeria Selective agar base (9L)	140	15.55 (50-80 plates)
Listeria Selective supplement	109	43.60 (50-80 plates)
Total for 25 samples		141.15
CHROMagar™ Listeria		
4x250mL CHROMagar™ Listeria (60 plates)	162	162.00 (60-70 plates)
Total for 25 samples		162.00

The cost for each medium was similar. However, if one considers the efficacy of each procedure and how this translates into labour costs, differences are discernable. For example, the samples in Lot 1 tested using the Australian/New Zealand Standard method took approximately 6-7 days to conclude that the *Listeria* spp. were detected and 7 –8 days for identification of a non-*L. monocytogenes* spp.. On the other hand, the adoption of CHROMagar™ Listeria agar plates eliminated non-*L. monocytogenes* spp. other than *L. ivanovii* and only took 4-5 days to conclude *L. monocytogenes* was not present. *L. ivanovii* is not commonly found in most foods (i.e 0.5%-2% as opposed to 41-71% *L. innocua*) thus the probability this bacteria would result in a false positive was limited.

The next factor to consider when comparing methods is the efficacy of each. The additional subculture step onto selective agar media at 24 h from the secondary broth, yielded an extra positive detection of *Listeria* spp., which would not have been detected by the Australian/New Zealand Standard method (Table 2.3). This result could have arisen due to other microbes initially present on the processed meat sample out-competing *L. monocytogenes* by completion of the 48 h incubation period of the second enrichment stage. Different selective plates were required to cover the variance in chemical make-up of food sample tested thus this table may demonstrate the need for more than two selective media per sample tested.

Presumptive *L. monocytogenes* isolates from Lot 3, were submitted for typing to a reference laboratory (see Section 2.2.2.4). Those isolates were identified as the same PFGE-type as the control and spiked samples within that Lot. Therefore it is likely that cross-contamination occurred during the processing of those samples. A contaminated pipette is hypothesised as the source of the contamination as the same 100μL pipette was used to transfer 0.1mL from half strength Fraser broth to 10mL of Fraser broth for all samples including spiked samples (Figure 2.1, Part 1). The false positives in this set of samples maybe due to cross-contamination from spiked samples to straight samples due to the pipette retaining contaminated fluid. This could be avoided by using a different pipette for control and samples, but also highlights the need for excellent aseptic technique and controls in testing laboratories.

The efficacy of the Australian/New Zealand Standard method (AS/NZS 1766.2.16.1:1998) for isolation of low levels of *L. monocytogenes* in naturally contaminated foods was also examined. Competition effects were noted in the enrichment broths when meat samples were spiked with varying levels of *L. monocytogenes*. In some cases the lower inoculum levels on spiked smallgoods samples were not detected on all selective plates. The potential effects of natural microflora are a continuing challenge for the food industry and require further study.

In conclusion, this survey suggests Australian smallgoods manufacturers have improved the control of *L. monocytogenes* in VP/MAP meats. A small percentage of other *Listeria* spp. were present mainly within the sliced meat section of the deli. The presence of other *Listeria* spp. on smallgoods products is indicative of poor cleaning and corrective action is required by industry to eradicate sources of contamination. Detection of *Listeria* spp. can be costly to industry due to large scale retesting of products.

3 Isolation and growth of Lactic acid bacteria in ham.

3.1 Introduction

Lactic acid bacteria (LAB) are a part of the natural microflora of vacuum packed (VP) and Modified Atmospheric Packaged (MAP) meat (Grau and Vanderlinde, 1992; Yang and Ray, 1994; Devlieghere *et al.*, 1998; Samelis *et al.*, 1998; Amezquita and Brashears, 2002; Mataragas *et al.*, 2002; Budde *et al.*, 2003). Amezquita and Brashears (2002) studied LAB as competitors against *L. monocytogenes* on ready-to-eat meats (RTE). 49 isolates were identified from 10 commercially available RTE meats, including processed ham. Six strains were inhibitory against *L. monocytogenes,* including *Pediococcus acidilactici*, *Lactobacillus casei* and *Lactobacillus paracasei.* From Amezquita and Brashears (2002), it was not clear which LAB were present on VP or MAP ham. The studies described in this Chapter required knowledge of the naturally occurring LAB on MAP ham. Samelis *et al.* (1998) reported that *Lactobacillus sake* and *Leuconostoc mesenteroides* spp. caused the greatest degree of contamination on vacuum packed cooked ham at 4 and 8°C. *Le. mesenteroides* spp. are commonly found on VP ham (Yang and Ray, 1994; Mataragas *et al.*, 2003a). Other commonly isolated strains on VP ham include *Leuconostoc carnosum* spp. (Yang and Ray, 1994; Bjorkroth *et al.*, 1998; Jacobsen *et al.*, 2003) and *Lb. sakei* spp. (Krockel, 2000; Katla *et al.*, 2001; Petaja *et al.*, 2003).

Biological control involves artificial control of pests and parasites by the use of other organisms. The three LAB above have been associated with *L. monocytogenes* inhibition and therefore may be considered as biological control agents against *L. monocytogenes* growth. Inhibition of *L. monocytogenes* by certain strains of LAB has been demonstrated in food (Duffes *et al.*, 1999a; Amezquita and Brashears, 2002) and broth systems (Winkowski and Montville, 1992; Huang *et al.*, 1993; Taranto *et al.*, 2000). The mechanism of inhibition was thought to be species specific and to relate to bacteriocin production by LAB (see Section 1.15.1) or non-specific relating to a mechanism other than bacteriocin production (see Section 1.6). Mataragas *et al.* (2003b) found *L. monocytogenes* was inhibited by *Le. mesenteroides*

(L124), and the inhibition was attributed to bacteriocin production. A later study used the bacteriocin of *Le. mesenteroides* L124 to inhibit *L. monocytogenes* in fermented sausages (Mataragas *et al.*, 2003a). That study involved using live cultures of *Le. mesenteroides* L124 and concentrated *Le. mesenteroides* L124 bacteriocin solution. The bacteriocin exhibited strong inhibition when pH was between 5-7. Bacteriocin production was also associated with *L. monocytogenes* inhibition by *Lc. carnosum* 4010 introduced at a level of 10^7 CFU.g^{-1} (Budde *et al.*, 2003; Jacobsen *et al.*, 2003). However, some *Lc. carnosum* are not suitable as biological control agents as they have been associated with spoilage problems in cooked VP ham products (Bjorkroth *et al.*, 1998). *Lb. sakei* has also been associated with inhibition of *L. monocytogenes* (Leroy and de Vuyst, 2001; Katla *et al.*, 2002) however, inhibition was associated with non-bacteriocin producing strains as well as bacteriocin producing strains (Katla *et al.*, 2001; Katla *et al.*, 2002).

In this chapter, naturally occurring LAB from MAP-RTE ham products were isolated. Products near the end of shelf life were selected to increase the likelihood of isolating LAB. The isolates were identified by using biochemical test strips (API *Listeria*, bioMérieux Inc., Appendix 2) and 16S rDNA sequence analysis, and tested for bacteriocin production. The growth response of selected LAB isolates to temperature was also characterised. Of the strains isolated, those with appropriate growth kinetics were used in trials investigating bacterial interactions between LAB and *L. monocytogenes* both in broth culture (see Chapter 4), and on commercially produced MAP ham (see Chapter 5).

3.2 Materials and methods

3.2.1 Sampling and Isolation procedure for Lactic Acid Bacteria:

Two MAP ham products from two different manufacturers were purchased from supermarkets in the local area:

Ham 1:
Product: "Champagne Ham"
Ingredients: pork meat, salt, soy protein, starch, sugars, food acid, mineral salts, antioxidant (316), nitrite, water added.
Days left to use-by-date: sampled on day of expiry.

Ham 2:
Product: "Leg Ham"
Ingredients: pork meat leg, salt, mineral salts, sugar, antioxidant (318), sodium nitrite (250), water added.
Days left to use by date: sampled seven days before use-by-date.

10 g of each ham sample was stomached in 90 mL of PW for 2 minutes. The sample was further diluted in PW and 100μL of the 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6} dilutions surface plated onto de Man, Rogosa, Sharp agar (MRS) using a spiral plater (Appendix 2). Duplicate plates of each dilution were prepared and incubated for 48 hours at 25°C and 37 °C.

Initially, individual, random colonies from plates were subcultured and incubated at 25°C for 1-2 days on TSA plates. The sampling methodology was changed, as the above method did not yield a representative sample. Thereafter, a specific area method was used to provide a better representation of the number of LAB on the ham surface. This was achieved by using a 2 cm square grid drawn on the surface of the 10^{-5} dilution plate. This dilution plate was selected as colonies were evenly separated on the agar surface. Each of the colonies within the nominated area was subcultured to TSA and incubated at 25°C for 1-2 days.

3.2.2 Identification of lactic acid bacteria isolates

Isolates that were gram-positive rods in chains or pairs, and catalase negative were further identified using commercial test kits for LAB and 16S rDNA sequence analysis.

3.2.2.1 Carbohydrate fermentation

A commercially available carbohydrate fermentation test kit was used, namely API strips (API 50 CH, bioMérieux Inc.) and pH indicator medium (API 50CHL, bioMérieux Inc.). Kits were used as *per* manufacturers' instructions and are summarised below.

Axenic cultures isolated as described in Section 3.2.1 were plated onto MRS for 24-48 h at 25°C or 37°C. A sterile cotton bud was used to harvest all the bacteria from the plate. The cotton bud was then placed into 2 mL of sterile water in a McCartney bottle and vortexed for 2 minutes to release all the cells. Sufficient volume of the suspension was added to a second tube of 5 mL distilled water to achieve "2" on the McFarland turbidity scale. The quantity used from the initial suspension to create a "2" McFarland turbidity level was noted. Double this quantity was added to API 50 CHL medium. The medium was carefully placed in the wells of prepared strips and was overlayed with sterile paraffin oil. The strips were incubated at 30°C as this temperature was intermediate for 25 °C and 37 °C.

API strips were assessed after 24 and 48 h incubation. Positive results were indicated by a colour change from purple to yellow. A score of 0 was given to purple wells and classified as a negative reaction. A score of 5 was given for yellow wells and classified as a positive reaction. Scores between 1 to 4 depended on the intensity of colour in the well. The profile obtained by these scores was entered into an identification software program and compared to the programs' database of known profiles (Appendix 2).

3.2.2.2 16S rDNA sequence analysis

Confirmed LAB isolates from API 50 CH testing were further identified by 16S rDNA sequencing to ensure the accuracy of the biochemical test kits. The

16S rDNA procedure was conducted initially by Dr John Bowman and subsequently on random samples by Mr. Guy Abell (University of Tasmania). The procedure used is described below.

The isolates were grown on TSB media for two days at 25°C. Biomass was removed (3-4 loopfuls) and suspended in 300 mL saline EDTA buffer (0.1 M NaCl, 0.1 M disodium EDTA, pH 8.1) in 1.5 mL Eppendorf tubes. Lysozyme was added to tubes (50 ml of 40mg/mL lysozyme in sterile distilled water) and tubes were mixed by brief vortexing and then incubated for 1 h at 37°C. 40 mL sodium dodecyl sulfate (40 mL of a 20% solution) was then added and tubes incubated at 55°C for 5 minutes. Lysates were deproteinated by the addition of 20 mL proteinase K (10mg/mL in sterile distilled water) with incubation at 37°C for 20 min. Further deproteination was achieved by addition of 100 mL of 5M sodium perchlorate. Tubes were briefly vortexed and then extracted with one volume of 24:1 chloroform-isoamylalcohol. The emulsion was vortexed and then centrifuged at 14,000 rpm for 5 min. The upper aqueous layer was then transferred to a new tube. The DNA in the aqueous phase was purified using the Prep-a-Gene kit (Biorad) according to the manufacturer's instructions. DNA obtained were eluted in 50 mL sterile deionised water and stored at –20°C.

PCR reactions were performed using 16S rDNA primers 519f (5'-CAG CMG CCG CGG TAA TAC-3') and 1492r (TAC GGY TAC CTT GTT ACG ACT T-3') which amplifies an approximate 1-kb fragment of the 16S rRNA gene. The PCR reactions were set up in 0.1 mL tubes with DNA (1-5 mL) and the primers (25 pmol/mL) added to sterile water to a total volume of 25 mL. To this was added an equal volume of HotStart PCR Mastermix (Qiagen). The tubes were then transferred to DNA Engine model PTC-200 thermocycler (MJ Research) and amplification was performed using the following program: cycle 1 – 95°C, 15 min.; cycles 2-36 – 94°C for 1 min., 50°C for 1min., 72°C for 1 min; cycle 37 – 72°C for 4 min; followed by a soak at 10°C.

PCR products were checked for correct size using agarose gel electrophoresis using 1% agarose gels prepared with TAE buffer (20 mM Tris-HCl, 50 mM sodium acetate and 1 mM disodium EDTA, pH 7.0) containing 1 mg/mL ethidium bromide. Gels were run in TAE buffer at 80 V for 30-40 min. Gels were examined using UV

transillumination. PCR amplicons were compared to a DNA molecular weight ladder (Hyperladder IV, Bioline). Bands of the correct size and sufficient DNA concentration were then purified for sequencing using the Prep-a-Gene kit (Biorad) according to the manufacturer's instructions. Purified PCR products were eluted in 30-50 mL sterile deionised water and stored at –20°C.

Sequencing reactions and subsequent purification were performed using the Quicktime DTSC cycle sequencing kit (Beckman-Coulter) according to the manufacturer's instructions. Sequence reactions were then analysed on the Beckman-Coulter CEQ2000 automated DNA sequencer. Sequences obtained were checked for accuracy using the BioEdit program (Hall, 1999) and then compared against the GenBank nucleotide database using BLAST-n searches (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi). Closest related species to the LAB isolates were determined by determination of the similarity of 16S rDNA sequences. It was considered if sequences were >99% similar, there was a high probability they belonged to the same species (Stackebrandt and Goebel, 1994).

3.2.3 Storage of LAB isolates:

LAB that were isolated using the above methods were required for a later study and thus required long-term storage as described in Appendix 1.2.2.

3.2.4 Bacteriocin assay:

Selected LAB isolates were assessed for bacteriocin production against five strains of *L. monocytogenes* (Scott A, L5/22, 20423, 20432 and 20425-see Appendix 1.1). A commercial strain of lactic acid bacteria, ALC01, a strain of *Lactobacillus plantarum* known to produce bacteriocin, was used as a positive control (Appendix 1.1). Variations of the agar spot method of Spelhaug and Harlander (1989) were used as follows. LAB were resuscitated from cryogenic storage by plating onto MRS agar and incubating at either 25 or 30°C for two days (some strains required a lower temperature of incubation to reduce time spent in the lag phase). A single colony was then inoculated from each plate into 10mL of MRS broth and incubated for 24 h at 30°C to provide stationary phase cultures. 5μL of each test culture was placed onto an MRS agar plate with spots spaced 3cm apart. The positive control

(Lb. plantarum) was included with each plate. Triplicate plates were prepared to test against each of the 5 *L. monocytogenes* strains. Plates were incubated for 48h at 30°C or until visible colonies appeared. After incubation, one of each triplicate set of plates was inoculated with a protease enzyme (alpha-chymotrypsin enzyme; Sigma, L-4129), i.e. only one protease enzyme was used. The protease enzyme was prepared by suspending the enzyme in 0.1 M sodium phosphate buffer (pH7) to a final concentration of 10mg.mL^{-1}. The enzyme should deactivate any bacteriocins produced by LAB. This method is a variation to the Spelhaug and Harlander (1989) procedure as 10μL of the protease enzyme was placed in small slits on either side of the spot colonies (i.e. 5 μL on either side of spot colony). The plates were then incubated for two hours at 30°C.

Prepared plates (i.e. containing visible LAB colonies +/- protease enzyme) were then overlaid with appropriate *L. monocytogenes* cultures as follows. Five flasks containing 21.6mL of sterile, soft BHA (0.7% g.L^{-1} agar) were prepared and maintained at 55°C. While a temperature of 47°C was specified by Spelhaug and Harlander (1989), this rendered the agar preparations difficult to work with as the agar often 'set' prior to application. Thus 55°C was used to ensure a smooth overlay plate. 2.6mL of a 10^{-5} dilution of stationary phase culture (24h at 37°C) of *L. monocytogenes* was added to a flask and mixed gently to provide a level of ~10^4 CFU.mL^{-1}. This was repeated for each strain. 8mL of inoculated agar was then poured over the surface of the control and enzyme treated plates. 8 mg of pyruvic acid was added to the remaining 8 mL of seeded agar and poured over the surface of the remaining prepared plate. Pyruvic acid reacts with peroxide, thus removing it from bacterial cells. All plates were incubated at 30°C for 24-48 h.

3.2.5 Temperature – Growth Rate Determinations

3.2.5.1 Inoculum preparation

LAB isolates were resuscitated from –70°C by allowing them to rapidly defrost at room temperature (~ 25-38°C). A loopful of cryogenic culture was streaked onto the surface of an MRS plate and incubated at 25°C for 24 h. A single colony from that plate was inoculated into 10 mL MRS broth and incubated for a further 20h to provide a stationary phase culture of ~10^9 CFU.mL^{-1}.

3.2.5.2 *Media preparation*

MRS broth was prepared and the a_w adjusted to 0.975 to simulate the average a_w in VP/MAP processed meats (Appendix 5, Tables 2 to 5). The a_w was adjusted using NaCl as the humectant, and a_w was determined from triplicate measurements using a dew-point water activity meter (Aqua Lab, CX-2; Appendix 2). The pH of the NaCl amended MRS broth was 5.5.

25mL of MRS at a_w 0.975 was added to L-tubes which were then autoclaved (121°C for 20 minutes). Prepared media were allowed to stand at room temperature for two days to ensure their sterility. This volume added to the L-tubes left little air space at the top of the tube and was done to prevent excessive oxygenation of the liquid. It was anticipated that this would assist in simulating an environment where oxygen is limiting such as VP/MAP processed meats.

3.2.5.3 *Construction of growth curves*

A temperature gradient incubator (Appendix 2) was used and consisted of 24 temperature ports (12 on each side of the bar). The temperature range was set to ~5-35°C. L-tubes were temperature equilibrated for 24 h prior to inoculation. Growth curves were constructed by measuring per cent transmittance (%T) at 540 nm with a digital spectrophotometer (Appendix 2). Initial %T measurements (i.e. prior to inoculation) were made for all tubes once temperature was stable. A scale of 0-50%T was used to monitor changes in %T. The reasons for using this scale as apposed to 100%T are explained in Section 3.2.5.4.

A small volume, 1 to 1.5mL, of stationary phase culture (Section 3.2.5.1) was added to L-tubes to obtain an initial transmittance of around 80-90% (i.e. equivalent on a 50%T scale of 43.5% to 45.0%). This is equivalent to an initial inoculum level of ~10^6 CFU.g^{-1}. The turbidity of the test broth was monitored, with measurement times chosen to correspond, approximately, to successive drops in transmittance of ≤5%. Each L-tube was monitored until %T ceased to change, assessed as three successive readings without change in %T. %T values were converted to OD values using Eqn. (3.1).

$$\text{Absorbance } \%T = 2 - \log_{10}(\%T) \qquad \text{(Eqn. 3.1)}$$

Estimates of generation and lag time for either absorbance or viable count data were analysed by linear regression as follows. Log_{10}(OD or VC) were plotted against time. Generation time was defined as the time for the bacterial population to double in cell numbers or turbidity. A straight line was fitted (Microsoft® Excel) to a visually selected set of points that appeared to represent the exponential phase of growth. Typically this region included ≥6 to 15 points. The regression line is of the form:

$$y = c + mx \qquad \text{Eqn. (3.2)}$$

where y = log numbers

c = the y axis intercept

m = the slope

x = time

The generation time is calculated by dividing the slope, m, by 0.301 (equivalent to $\log_{10}2$), thus:

$$GT = \frac{0.301}{m} \qquad \text{(Eqn. 3.3)}$$

In this study lag time is depicted by the value on the x axis where the intercept of the regression line through the exponential part of the growth curve equals the starting y value. Lag time may be calculated by:

$$\text{Lag} = \frac{y\ (\text{initial}) - c}{m} \qquad \text{(Eqn. 3.4)}$$

Generation time estimates were converted to growth rate, "GR", (the reciprocal of generation time). The square-root of GR was plotted against temperature to determine the T_{min} values for each LAB strain. Estimates for growth rate of each LAB were compared to estimates from appropriate predictive models (Devlieghere *et al.*, 1998; Leroy and de Vuyst, 2001).

3.2.5.4 Calculation of T_{min}

T_{min}, the notional temperature at which growth rate is considered equal to zero, was calculated as follows. For linear data, i.e. no data above the optimum for temperature was available, T_{min} is calculated by plotting the square root of (1/GT) against temperature and finding where the regression line bisects the x axis (Eqn 3.5).

$$\text{sqrt } (1/GT) = mx + c. \qquad \text{(Eqn 3.5)}$$

For non-linear data, i.e. containing data above and below the optimum, a different model is required. The 4 parameter square-root model of Ratkowsky et al (1983). Equation 3.6, is fitted using the curve fitting program Ultra-Fit v.3.0.5 (Biosoft©).

$$\sqrt{\text{rate}} = b*(\text{temperature}-Tmin)*(1-\exp(c*(\text{temperature} - Tmax))) \qquad \text{Eqn (3.6)}$$

3.2.5.5 Media colour variations

A post-experimental procedure was conducted to assess the validity of the data measured using a spectrophotometer scale of 50%T as compared to 100%T, and to compare turbidimetric readings to Total Viable Count (TVC) data. The MRS broth was a dark colour, probably due to sugar caramelising during sterilisation. The high sugar content of MRS broth ($40g.L^{-1}$) and the addition of salt may have attributed to the observed darker colour. The T% readings of the darker broth were reading as errors on the digital scale spectrometer due to the wavelength selected (i.e. 540 nm) not being high enough. The 'zero' was lowered to 50%T so dark coloured broth could be measured on a less sensitive measuring scale as compared to the digital scale. In addition to this, the spectrophotometer used can only measure on a scale between 0 to 100% thus any scratches on tubes measuring above this range were not measuring at 100%T. Thus 50%T was also adopted to include %T values higher than 100 to measure scratched glass and growth rate of each bacteria. According to Beers law, the %T is calculated by the following Eqn. (3.7) (Chang, 1981). This equation only accounts for 100%T, thus 50%T may have been questionable.

Therefore further trials were conducted to compare 100%T readings with 50%T readings.

$$T = I/I_o \times 100 \qquad \text{Eqn. (3.7)}$$

T = transmittance

I = incident radiation

I_o = transmitted radiation

3.2.5.6 Growth of LAB at 20°C and 30°C at 100%T:

Measurements made using a scale of up to 50%T may not be as accurate as a scale of up to 100%T. Duplicate samples were carried out using turbidity measurements at 100%T and Total Viable Count (TVC) at the two temperatures 20°C and 30°C. These results were compared to turbidity measurements at 50%T to allow for any error in growth rate calculations when using 50%T.

3.3 Results

3.3.1 LAB isolated from MAP ham.

The first sample (Ham 1) contained 22 colonies in the 2 cm reference square (at 25°C) of the 10^{-5} dilution and the plate count on MRS agar was 2.7×10^{6} CFU.g^{-1}. Of those 22 isolates, 8 isolates were randomly chosen for identification. The second sample (Ham 2) yielded 15 isolates in the 2 cm reference square (at 25°C) of the 10^{-5} dilution and the plate count on MRS agar was 1.58×10^{6} CFU.g^{-1}. Of those 15 isolates, only 12 were identified due to the limited availability of commercial test strips. Thus, 20 isolates from MAP ham were identified using API and 16S rDNA analysis and are listed in Table 3.1.

Gram stains (Appendix 4.1) and the fermentation profiles of the API tests were used initially to identify LAB isolates. Random isolates with high API % significance levels were selected for confirmation using 16S rDNA analysis as the API identifications were assumed to be correct. A follow up identification of isolates using 16S rDNA analysis was conducted using random isolates to ensure accurate identification of isolates.

Table 3.1 Identification of lactic acid bacteria isolated from MAP ham

	37°C	Morphology	API Test Kit		16S rDNA analysis	
			Identification	%sig*	#1	#2
Ham 1						
1	+	cocci, cluster	*Le.mes.mes.dext*	99.9	Not tested	*Le. mesenteroides sub.sp mesenteroides*
2	+	cocci, cluster	*L.mes.mes.dext*	99.9	Not tested	*Le. mesenteroides* strain NRIC 1539
3	+	cocci, cluster	*Le.mes.mes.dext*	99.9	Not tested	*Le. mesenteroides* strain NRIC 1539
4	+	cocci, cluster	*Le.mes.mes.dext*	99.9	Not tested	*Le. mesenteroides* strain NRIC 1539
5	+	cocci, cluster	*Le.mes.mes.dext*	99.9	Not tested	Not tested
6	+	rods in pairs	*Lb.curvatus*	84.3	Not tested	Not tested
7	+	long chains, small rods or cocci	*Le.mes.mes.dext*	66.7	Not tested	*Lb. sakei*
8	+	long chains, short rods or cocci.	*Le.mes.mes.dext*	66.7	Not tested	Not tested
Ham 2						
1	+	small rods almost cocci, chains	*Lb.delb.delb*	98.7	*Lc. carnosum*	Not tested
2	+	small rods/cocci in chains and clusters	*Stra.saliv.thermoph*	95.3	*Le. mesenteroides*	Not tested
3	−	small rods in pairs	*Leuco.mes.mes/dext*	98.3	*Lb. curvatus (DSM20010) or Lb. sakei*	Not tested
4	+	small rods in pairs	*Lb. fermentum*	95.8	*Lb. curvatus (DSM20010) or Lb. sakei*	*Lb. sakei*
5	−	small rods or cocci in chains	*Lb.delb.delb*	88.1	Possible *Lc. carnosum*	*Lc. carnosum*
6	−	very small rods or cocci in chains/clusters	*Lb.delb.delb*	91.7	*Lc. carnosum*	*Lc. carnosum*
7	−	small rods or cocci in chains and clusters	*Le.mes.mes/dext*	97.8	*Lc. carnosum*	Not tested
8	−	coccoid, clusters	*Lb.delb.delb*	91.7	*Lc. carnosum*	*Lc. carnosum*
9	−	coccoid clusters	*Lb.delb.delb*	91.7	*Le. mesenteroides*	Not tested
10	−	coccoid clusters	*Lb.delb.delb*	91.7	*Lc. carnosum*	*Lc. carnosum*
11	−	small rods in pairs.	*Le.mes.mes/dext*	96.3	Not tested	*Lc. carnosum*
12	−	rods, pairs and chains	*Lb.curvatus*	99.5	contaminated	*Lb. sakei*

% significance: The significances level of the set of results being the species indicated.
Shaded cells represent similar species identification for both technique

Of the 20 isolates listed in Table 3.1, only 4 were identified as the same species, *Le. mesenteroides*, by both API and 16s rDNA analysis. The latter method of identification is considered more accurate (Amezquita and Brashears, 2002).

Similar types of bacteria were found on both ham samples. Ham (1) showed a different isolate ratio to Ham (2). Ham (1) supported the growth of 62.5% (5 out of the 8 isolates) of *Le. mesenteroides* species and the rest of the isolates may have been *Lb. sakei* or more *Le. mesenteroides* species. Those species were less well defined due to lower % significance results. Ham (2) supported only 16% (2 out of the 12 isolates) *Le. mesenteroides* species, 25% (3 out of the 12 isolates) *Lc. carnosum* and 58% (7 out of the 12 isolates). When using the API carbohydrate fermentation method to identify LAB isolates, the dominant species on Ham (2) were the isolates *Lactobacillus delbrueckii. delbrueckii (*isolates 5, 6, 8,9 and 10) . When 16s rDNA analysis was used, these isolates were identified as *Le. mesenteroides* or *Lc. carnosum.* All strains from Ham (1) sample grew at 37°C whereas 80% of the strains from ham (2) showed no growth at 37°C but all grew at 25°C.

Isolates 1 (Ham 1), 10 (Ham 2) and 12 (Ham 2) were selected for subsequent studies as they occurred most frequently on both ham products and were thus considered representative of common LAB on MAP ham. They were identified as *Lb. sakei*, *Le. mesenteroides* and *Lc. carnosum* respectively. Each strain was characterised further for bacteriocin production and their growth response to temperature (see below).

3.3.2 Bacteriocin production:

Table 3.2 reports bacteriocin production of the 3 LAB isolates from ham and the commercial anti-listerial LAB against various strains of *L. monocytogenes.* A strain was considered to be a bacteriocin producer if overlay plates showed a clearing zone around the LAB colony not treated with protease (i.e. the control), no zone around the protease treated colony and a zone around the pyruvic acid treated colony. A strain was considered to be a H_2O_2 producer if a zone was evident around the protease treated and the control colonies, and no zone was evident around the pyruvic acid treated colony.

Table 3.2 Results for a bacteriocin assay of four strains of lactic acid bacteria against five strains of *Listeria monocytogenes*.

Strain	Lactic acid bacteria	Treatment		
		+ protease enzyme	overlay only	+pyruvic acid
L. monocytogenes Scott A	*Le. mesenteroides*	–	–	–
	Lc. carnosum	–	–	–
	Lb. sakei	–	–	–
	Control- *Lb. plantarum* ALC01	+	+	–
L. monocytogenes L5/22	*Le. mesenteroides*	–	–	–
	Lc. carnosum	–	–	–
	Lb. sakei	–	–	–
	Control- *Lb. plantarum* ALC01	–	+	+
L. monocytogenes 20423	*Le. mesenteroides*	–	–	–
	Lc. carnosum	–	–	–
	Lb. sakei	–	–	–
	Control- *Lb. plantarum* ALC01	–	–	–
L. monocytogenes 20432	*Le. mesenteroides*	-	+	–
	Lc. carnosum	-	+	–
	Lb. sakei	–	–	–
	Control- *Lb. plantarum* ALC01	–	–	–
L. monocytogenes 20425	*Le. mesenteroides*	-	+	+
	Lc. carnosum	-	+	-
	Lb. sake	–	–	-
	Control- *Lb. plantarum* ALC01	–	–	–

-Shaded cells indicate bacteriocin production

Of the strains tested, 2 LAB strains produced bacteriocin against 2 of the *L. monocytogenes* strains (Table 3.2; shaded cells). More specifically, *Lb. plantarum* ALC01 (the commercial anti-listerial strain used for the positive control) produced bacteriocins against one strain, *L. monocytogenes* L5/22. H_2O_2 production was also observed against *L. monocytogenes* Scott A by the control strain. *Le. mesenteroides* produced bacteriocins against *L. monocytogenes* 20425. Atypical results were observed for some strains. Zones of inhibition were evident for *Le. mesenteroides* against *L. monocytogenes strains* 20432 and 20425 on the control plate only. A similar result was evident for *Lc. carnosum* and *L. monocytogenes* 20425. No firm conclusions could be determined from these data.

No LAB strains produced bacteriocins against *L. monocytogenes* strains Scott A, L5/22 and 20423 to be used later in this study (i.e. for Chapter 4 and 5). *Lb. sakei* did not produce bacteriocins against any of the 5 *L. monocytogenes* strains.

3.3.3 Generation time and T_{min} for LAB.

The temperature range of L-tubes for the temperature gradient bar run was 5°C to 31.8°C for *Le. mesenteroides*, 6.1°C to 32.2°C for *Lc. carnosum* and 4.7°C to 32.1°C for *Lb. sakei*. Estimates of generation time for temperatures within these ranges were calculated as described previously (Section. 3.2.5.3).

Square root plots for viable count and absorbance data for each strain are presented as Figures 3.2 to 3.4. *Lc. carnosum* required a 4 parameter square root model (Eqn. 3.6) to calculate the T_{min} value *carnosum* (Figure 3.1) whereas *Le. mesenteroides* (Figure 3.3a) and *Lb. sakei* (Figure 3.3b) required the only the two parameter square root model (Eqn. 3.5).

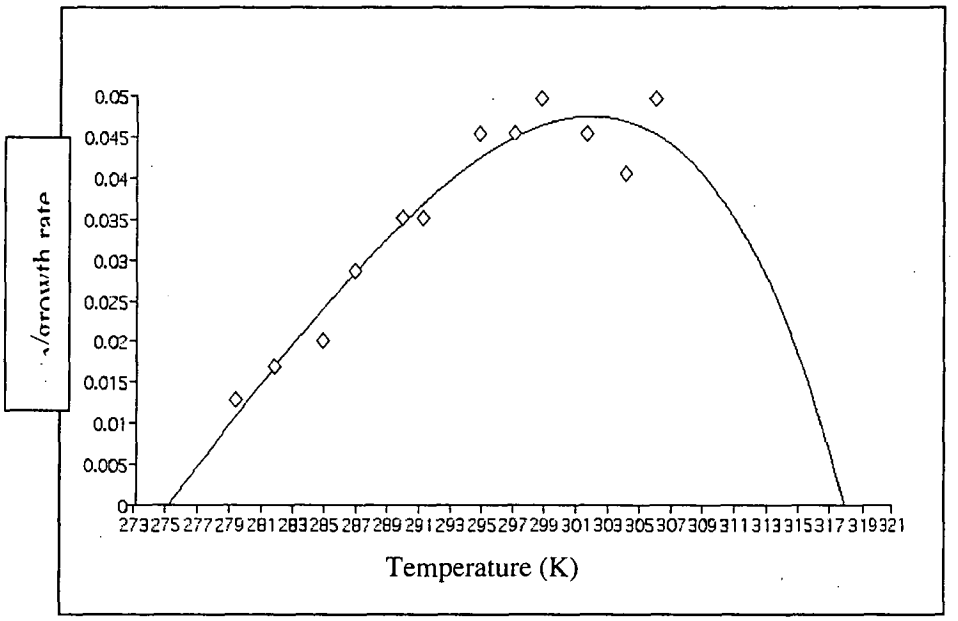


Figure 3.1 The fitted curve for estimation of T_{min} for *Lc. carnosum* using the 4 parameter square root model of Ratkowsky et al. (1983).

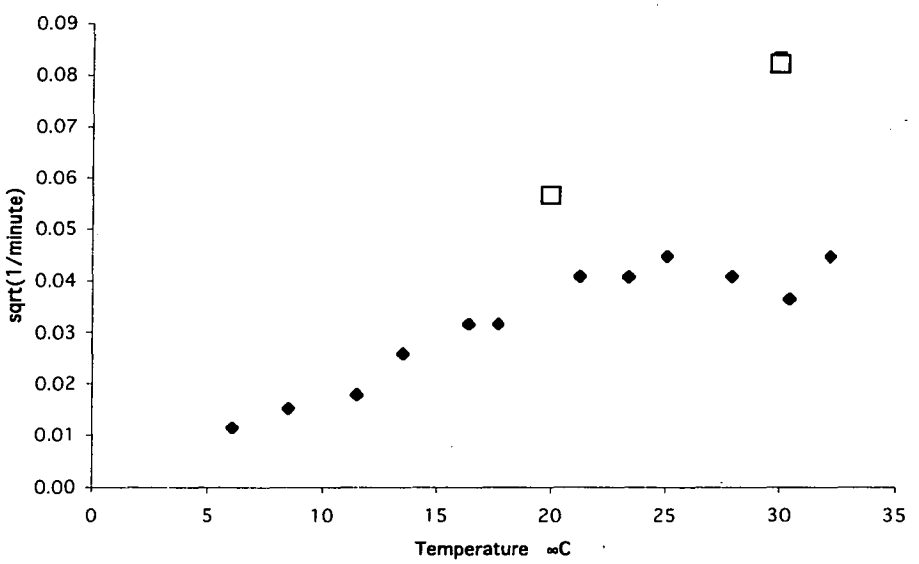


Figure 3.2 Plot of square root (1/GT) versus Temperature for *Lc. carnosum* determined by absorbance (◆) and duplicate viable count data at 20 and 30°C (□).

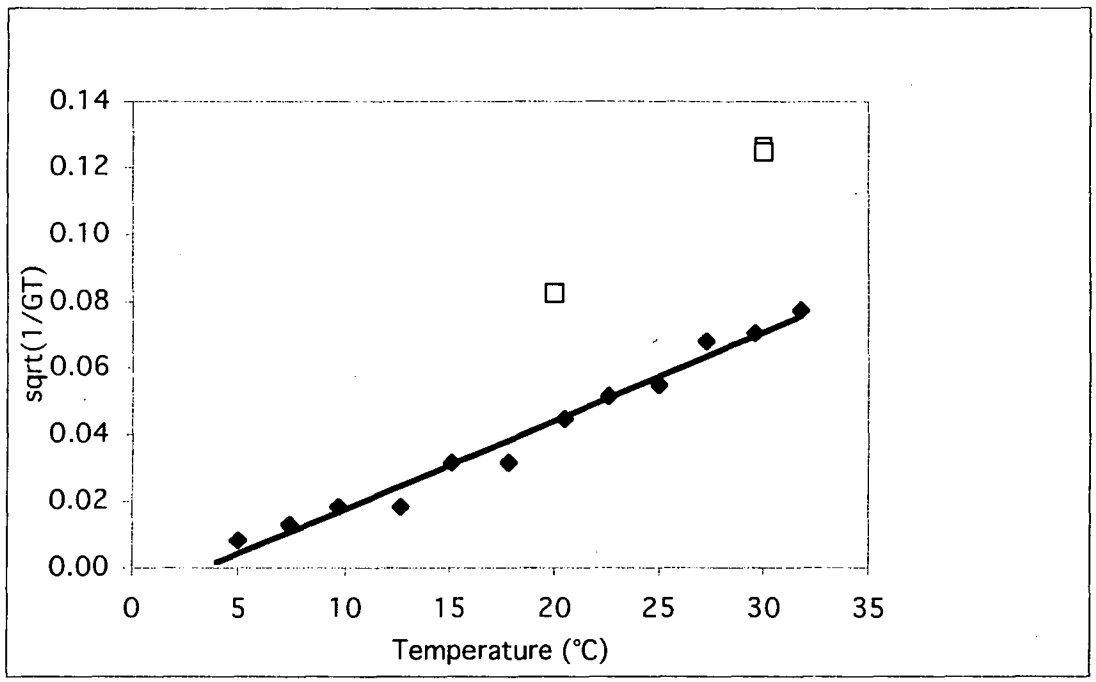


Figure 3.3a Plot of square root (1/GT) versus Temperature for *Le. mesenteroides* determined by absorbance (◆) and duplicate viable count data at 20 and 30°C (□).

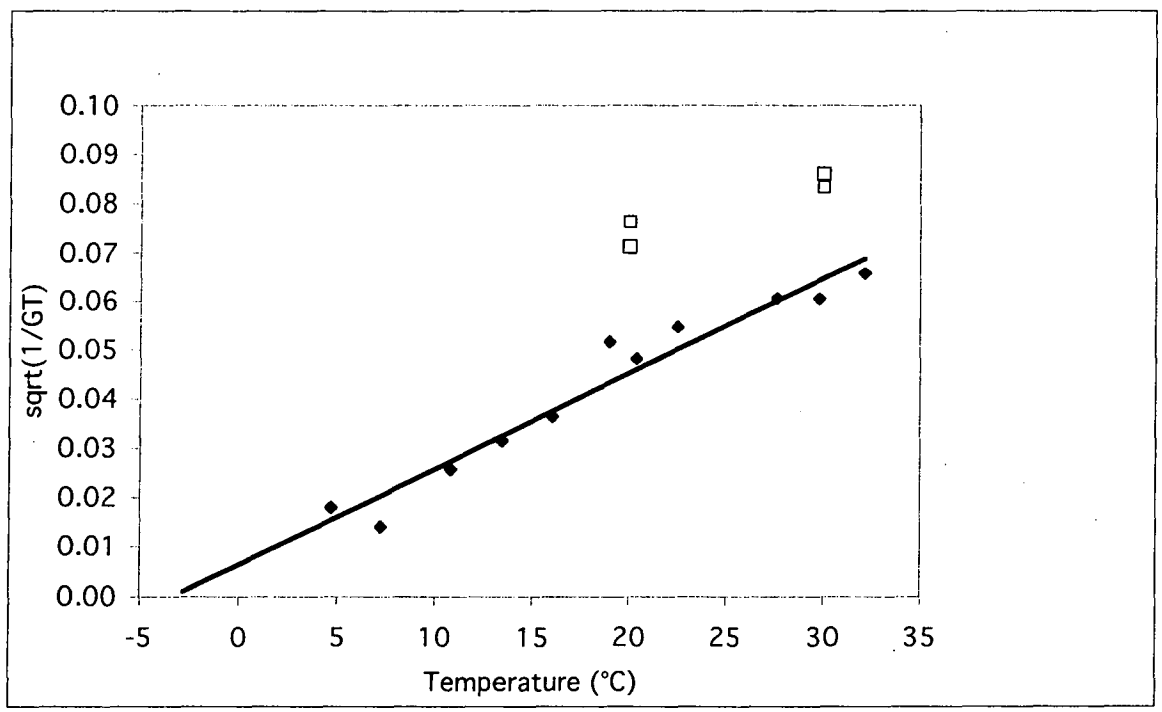


Figure 3.3b Plot of square root (1/GT) versus Temperature for *Lb. sakei* determined by absorbance (◆) and duplicate viable count data at 20 and 30°C (□).

Table 3.3 lists T_{min} estimates (calculated as described in Section 3.2.5.4) for the three LAB and *L. monocytogenes*. The T_{min} for *Lb. sakei* was lower than the other two LAB and *L. monocytogenes*. The T_{min} estimates from Ross Model 1 (T. Ross, *pers. comm.*,) were compared to this study's T_{min} for conditions of water activity (i.e. a_w 0.975), pH and lactic acid content relevant to ham.

Table 3.3 The T_{min} values estimated for bacteria used in this study and for *L. monocytogenes* derived from other literature.

Organism	Strain	T_{min} (°C)	Reference
Le. mesenteroides	Not known	3.31	From this study
Lb. sakei	Not known	-3.96	From this study
Lc. carnosum	Not known	1.93	From this study
L. monocytogenes	Scott A	- 0.108±0.5°C	(Ross Model 1, a_w 0.975, pH 6.2, lactic acid (80 mM)*
L. monocytogenes	Scott A	1.5±0.5°C	(Nichols *et al.*, 2002)
L. monocytogenes	Scott A	1.43±0.761	(Tienungoon, 1998)

Ross Model 1 (GT(h)= /((pH-5.5)(water actvity-0.923)*((temperature +0.29)*0.1905)^2))))

Turbidimetric data were generated for the 3 LAB using the 50%T scale as discussed in Section 3.2.5.5. To assess the validity of growth rate estimates and T_{min} estimated from those data, LAB generation times were also measured at 20°C and 30°C turbidimetrically using the full scale of 0-100%T. These two temperatures were used to generate a rough estimate of T_{min} by forming a line through the points once the growth rates have been converted to sqrt (1/GT)). The sqrt (1/GT) points for these measurements were very close to the same temperature points at 0 to 50%T (data not shown). In contrast, the growth rate measured using TVC at 20°C and 30°C, showed sqrt (1/GT) points well above turbidimetric estimates at the same temperature. There was little variation between sqrt (1/GT) duplicate values measured using TVC for *Lc. carnosum* and *Le. mesenteroides*, thus these points were used together with T_{min} from Table 3.3 to form a linear regression line in a plot of sqrt (1/GT) against temperature (see Figures 3.4 to 3.6).

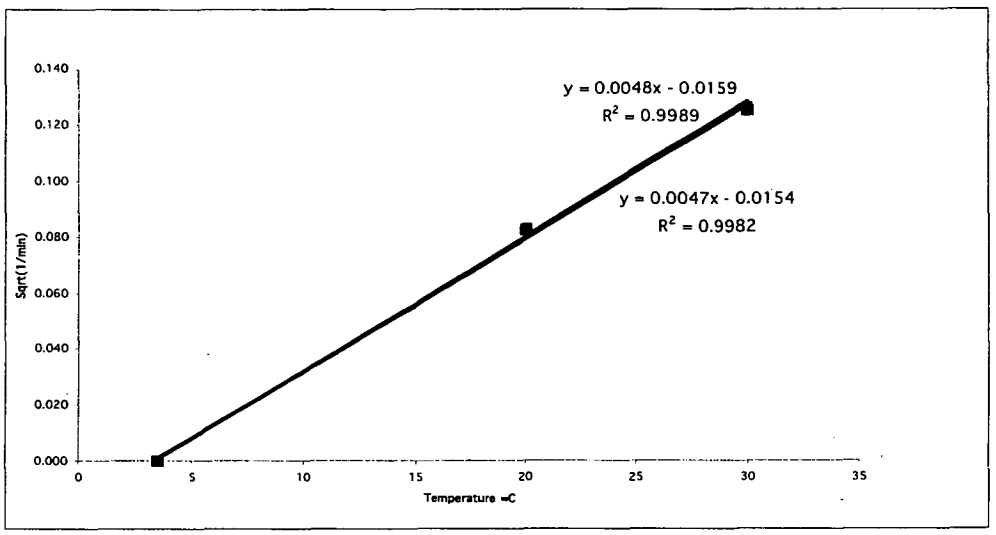


Figure 3.4 The square root of 1/GT (mins) against Temperature for *Le. mesenteroides* for growth at 20°C and 30°C and a T_{min} of 3.5°C, where (■) is the TVC points for *Le. mesenteroides.*

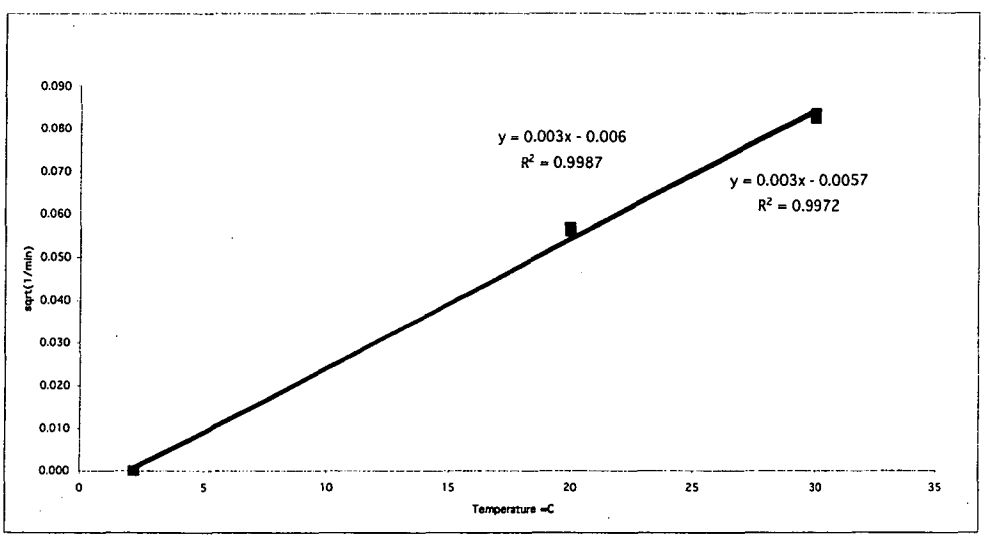


Figure 3.5 The square root of 1/GT (mins) against Temperature for *Lc. carnosum* for growth at 20°C and 30°C and a T_{min} of 2.2 °C where (■) is the TVC points for *Lc. carnosum.*

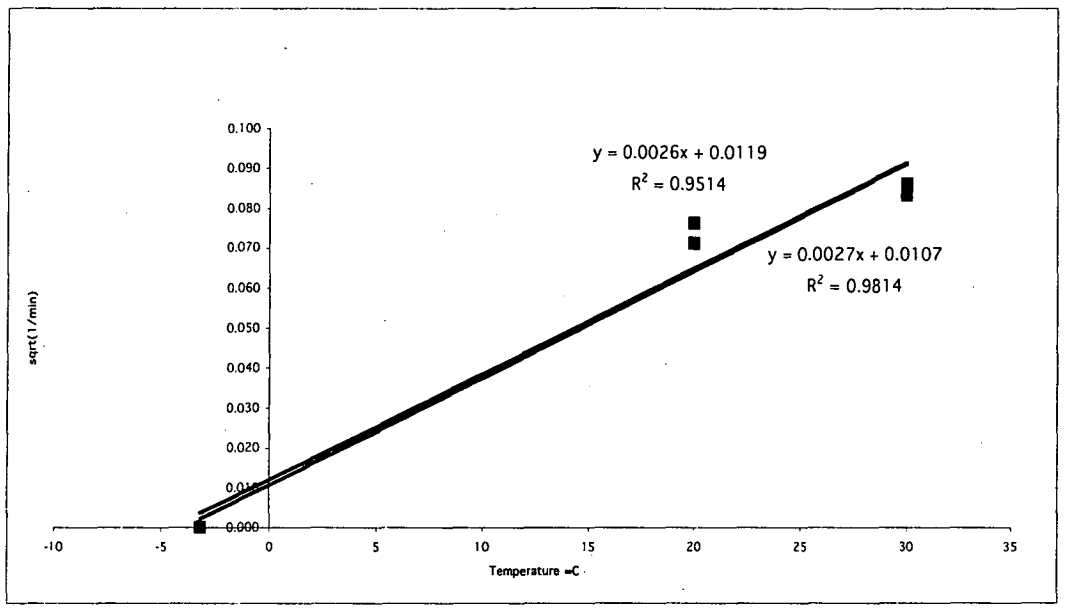


Figure 3.6 The square root plot for *Lb. sakei* for growth at 20°C and 30°C and a T_{min} of –3.2 °C where (■) is the TVC points for *Lb. sakei*.

In subsequent studies (see Chapter 5) estimates of GTs for LAB at temperatures of 4°C and 8°C were required, thus GT at these temperatures were extrapolated from the equation described above. The generation times derived from TVC measurements for *Le. mesenteroides*, *Lc. carnosum* and *Lb. sakei* are displayed in Table 3.4. These results are compared to *L. monocytogenes* growth rates from the model of Tienungoon (1998) at 4, 8, 20 and 30°C and conditions similar to vacuum packed ready-to-eat products (i.e. pH 5.5 to 6.5 and a_w 0.970 to 0.975, Appendix 5, Table 2 to 5).

The only LAB with a faster growth rate than *L. monocytogenes* at 4°C and 8°C was *Lb. sakei*. However, the growth rate was higher or the same for *L. monocytogenes* compared with LAB at 20°C and 30°C as shown in Table 3.4. Growth rates of other LAB estimated from predictive models developed by Wijtzes *et al.* (2001), Devegliehere *et al.* (1998) and Leroy and de Vuyst (2001) were similar to those found for *Lb. sakei* (Table 3.4 and Figure 3.7)

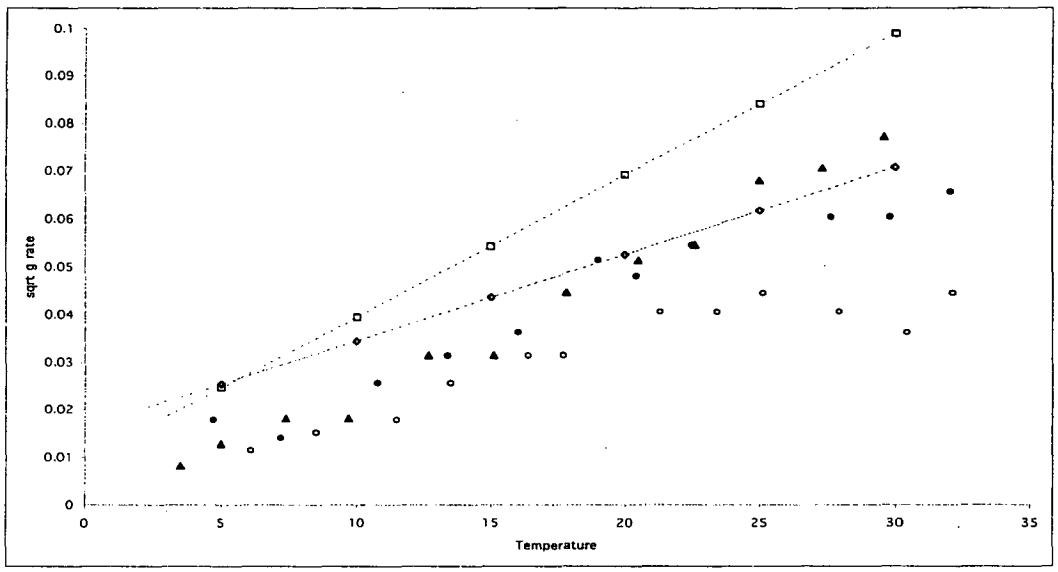


Figure 3.7 Comparisons of square root plots of LAB at 0.970, pH 5.5, where (□) is *Lb. curvatus* from predictive model of Wijtzes *et al.* (2001), (◈) is *Lb. sake* from predictive model of Devegliehere *et al.* (1998), (▲) is *Lb. sakei*, (○) is *Lc. carnosum* and (●) is *Le. mesenteroides*.

Table 3.4 Generation times calculated from TVC results for lactic acid bacteria grown in MRS broth adjusted to a_w 0.975, pH 5.5 and compared to literature data.

Lactic acid bacteria	4 °C	8 °C	20 °C average	30 °C average
Le. mesenteroides	1485.0 h	33.0 h	2.6 h	1.0 h
Lc. carnosum	440.0 h	50.0 h	5.7 h	2.4 h
Lb. sakei	34.0 h	15.0 h	4.1 h	2.0 h
Predictive model, *Lb. curvatus*, (Wijtzes *et al.*, 2001).	30.0 h	13.0 h	3.0 h	1.5 h
Predictive model, *Lb. sake* (Devlieghere *et al.*, 1998).	32 h	19 h	6.0 h	4.0 h
Logistic model *Lb. sakei*, (Leroy and de Vuyst, 2001).			2.2 h (25°C, pH 6.5)	
L. monocytogenes, pH 5.5, a_w 0.975*	124.0 h	24.0 h	3.3 h	1.4 h
L. monocytogenes, pH 6.5, a_w 0.975*	90.4 h	17.3 h	2.4 h	1 h

*Estimates for *L. monocytogenes* grown in TSB-Ye broth (Tienungoon, 1998).

3.4 Discussion

Identification of LAB via the API method did not appear to be efficient when results were compared to 16S rDNA analysis (Table 3.1). Molecular techniques such as pulse-field gel electrophoresis (PFGE), have been considered the best method for molecular characterization of LAB (Amezquita and Brashears, 2002). 16s rDNA sequence analysis was used by Amezquita and Brashears (2002) to confirm the type of LAB isolates after they had been identified by the API system. Bjorkoth *et al.* (1998) stated that differentiation between *Lc. carnosum* and *L. joelidium* must involve DNA testing as sugar fermentation is of little value. The varying results between the 16S rDNA analysis and API method of identification, may therefore suggest that API for lactic acid bacteria identification is not very accurate. A 16S rDNA analysis should be run in conjunction with the API tests. In these trials it was difficult to separate *Lb. sakei* and *Lb. curvatus* via API test strips and 16S rDNA analysis profiles. The two species are related phenotypically and genotypically however, they could be separated into groups using RAPD (Randomly Amplified Polymorphic DNA) (Berthier and Ehrlich, 1999), when biochemical analysis was used. This may explain why the growth rates of *Lb. sakei* from this study and those reported for *Lb. curvatus* (Wijtzes *et al.*, 2001) are similar (Table 3.4).

The three most common LAB found on MAP RTE ham in this study were *Le. mesenteroides*, *Lc. carnosum* and *Lb. sakei*. Similar findings have been reported on other RTE meats (Yang and Ray, 1994; Krockel, 2000). A survey by Krockel (2000) was undertaken on RTE meats one week past the "best before date" in a German food survey. The phenotypic and genotypic characterization of representative LAB isolates of selected samples showed that the dominant LAB species included *Lb. sakei* (54%), *Lc. carnosum* (23%), *Lb. curvatus* (15%), *Weissella viridescens* (8%) and *Carnobacterium piscicola* (8%) for all samples containing at least 10^5 CFU.g^{-1} LAB (Krockel, 2000).

Competition of *Lb. sakei*, *Le. mesenteroides* and *Lc. carnosum* against *L. monocytogenes* has not been as widely studied as the well documented bio-control bacterium *C. piscicola* (Schillinger and Holzapfel, 1990; Buchanan and Bagi, 1997;

Leroi *et al.*, 1998; Paludan-Muller *et al.*, 1998). The level of *C. piscicola* (8%) on meat was low in Krockel's (2000) report compared to higher levels of *Lb. sakei* (54%). The low presence of *C. piscicola* may suggest this is not the dominant LAB on some processed meat. The higher level of the three dominant LAB mentioned above, *Lb. sakei* (54%), *Lc. carnosum* (23%), *Lb. curvatus* (15%) may have an influence on *L. monocytogenes* suppression and thus have a role in the mechanism of the "Jameson Effect" which will be considered in greater detail in Chapter 4.

Lb. sakei had a faster growth rate than *L. monocytogenes* in this study and thus may be considered as a potential bio-control bacteria on VP or MAP processed meats. This result is supported by Krockel (2000) who reported that *Lb. sakei* was the dominant LAB on the meat product studied as it was the faster growing bacteria and had a greater inhibitory effect on *L. monocytogenes* due to its yield (analogous to the MPD) as opposed to other factors. Conversely, while the inhibitory effect of *C. piscicola* against *L. monocytogenes* has been well documented, it is not recognised as the main competitive bacterium on meats other than fish (Schillinger and Holzapfel, 1990; Buchanan and Bagi, 1997; Leroi *et al.*, 1998; Paludan-Muller *et al.*, 1998).

Generation times for *L. monocytogenes* growing at conditions relevant to VP/MAP meat were calculated using predictive models and are displayed in Table 3.4. Eqn. 3.8 for *L. monocytogenes* growth (Tienungoon, 1998) closely matched the broth system for LAB in this study.

$$\sqrt{rate} = 0.150*(T-0.88) * (1-\exp(0.536*(T-41.4))) * \sqrt{(aw-0.923)} * \sqrt{1-10^{4.97-pH}} * \sqrt{1-([LAC]/(3.79*(1+10^{pH-3.86})))} \quad \text{(Eqn. 3.8)}$$

The pH was not altered by lactate in the MRS broth in this study thus, this equation took into account the pH change due to HCl as opposed to lactic acid. Equation (3.8) cancels out the lactic acid content of the system. However VP/MAP ham products are known to have lactic acid present from native micro-biota and /or lactic acid production due to rigor mortis of the carcass. The content of lactic acid in vacuum

packed ham was 0.45±0.08% (w/w) in a study by Devlieghere *et al.* (2001). This would equate to 225mg/50g of ham product. Lactate content can be substituted into equation (3.8). This amount of lactate created an insignificant change in growth rate for *L. monocytogenes*. Growth rates in these two broths were tested in Chapter 4 and found to be similar thus these results could be compared. However the broths used for LAB and *L. monocytogenes* growth (Tienungoon, 1998) were MRS and TSB-Ye respectively thus this may affect the ability to compare actual results with model results.

The faster growth rate of LAB compared to *L. monocytogenes* at low temperatures has been well documented (Grau and Vanderlinde, 1992; Duffy *et al.*, 2000; Leroy and de Vuyst, 2001). This is contradicted by the results of this study where *Le. mesenteroides* and *Lc. carnosum* grew only half as fast as *L. monocytogenes* at 4°C and 8°C. In this study the fastest growing LAB at 4°C and 8°C was *Lb. sakei*. At 4°C, *Lb. sakei* was 2 to 3 times faster than *L. monocytogenes*. Faster growing bacteria are able to reach MPD more rapidly and thus prevent growth of all other bacteria once stationary phase is achieved. The generation time for *Lb. curvatus* (Wijtzes *et al.*, 2001) and *Lb. sake* (Devlieghere *et al.*, 1998) were similar to generation times of *Lb. sakei* found in this chapter (Table 3.4). This additional information supports the potential of *Lb. sakei* as a bio-control treatment against *L. monocytogenes*. Future work could involve determining the non-spoilage capabilities of *Lb. sakei*.

The benefit of knowing the T_{min} values for LAB and *L. monocytogenes* becomes apparent when considering which LAB would be competitive with *L. monocytogenes* in a cold storage system. Bacteria which can grow faster than *L. monocytogenes* at lower temperatures are suitable competitors for the pathogen. *Lb. sakei* appeared to have the lowest T_{min} value, was able to grow rapidly at 4°C and 8°C and thus may out-compete *L. monocytogenes* at the same temperature (see Chapter 5).

The LAB investigated in this study were shown to be bacteriocin negative against *L. monocytogenes* Scott A, L5/22 and 20423. The positive control *Lb. plantarum* produced bacteriocins against *L. monocytogenes* L5/22 and H_2O_2 against

L. monocytogenes Scott A, which confirms the validity of the assay. *Le. mesenteroides* was shown to produce bacteriocins against *L. monocytogenes* 20425. Consequently, of the strains to be used in the later challenge study (Chapter 5), one out of five *L. monocytogenes* strains is inhibited by 1 out of three LAB. Inhibition was observed against *L. monocytogenes* 20432 and 20425 by *Le. mesenteroides* and by *Lc. carnosum* against strain 20425. However, this was observed only on the control plate. No positive reactions were seen in protease treated colonies or pyruvic acid treated colonies thus, it is unlikely that the inhibition occurred due to either bacteriocins or H_2O_2 production. The non-specific inhibition of *L. monocytogenes* by LAB on vacuum packed ham is examined in Chapter 5 by using a greater ratio of non-bacteriocin producing LAB strains against *L. monocytogenes*.

Some reports relate the inhibition of *L. monocytogenes* on meat and in broth systems to the bacteriocins produced by LAB (Duffes *et al.*, 1999a; Katla *et al.*, 2001; Szigeti, 2001; Amezquita and Brashears, 2002; Katla *et al.*, 2002; Mataragas *et al.*, 2002; Jacobsen *et al.*, 2003). Katla (2001; 2002) examined the bacterial interactions between *Lb. sakei* and *L. monocytogenes* on vacuum packed smoked salmon and chicken cold cuts. Non-bacteriocin producing and bacteriocin producing strains (sakacin P) of *Lb. sakei* were added to meat at a level of 10^5 to 10^6 CFU.g^{-1}. Interestingly, both strains inhibited *L. monocytogenes* thus suggesting inhibition by *Lb. sakei* may not be via bacteriocin production. There was no bacteriocin produced by *Lb. sakei* against any of five strains of *L. monocytogenes* strains tested in this study. No LAB were inhibitory against *L. monocytogenes* Scott A which is the strain to be used in competition studies with *Le. mesenteroides* and *Lb. sakei* in Chapter 4.

H_2O_2 production by the LAB was also examined in this chapter. Pyruvic acid was added to medium to remove H_2O_2. In a biological system, catalase will catalyse this reaction. Pyruvic acid is associated with the breakdown of H_2O_2 in the medical field (Mazzio and Soliman, 2003). There was H_2O_2 production by *Lb. plantarum* against *L. monocytogenes* (Scott A). This was also seen by (Dominguez *et al.*, 1987) who reported H_2O_2 as an inhibitor of *L. monocytogenes* when temperatures were above 4°C. Dominguez et al (1987) treated milk with 0.495% H_2O_2 and added 10^7 CFU.mL^{-1}

of *L. monocytogenes* into the milk. The *L. monocytogenes* population increased after 24 h treatment with H_2O_2thus indicating the H_2O_2 may have been utilised.

4 Mechanisms of interactions between cells of different bacterial species in co-culture.

4.1 Introduction

Specific factors such as bacteriocins are reported as a cause of inhibition of one bacterium by another as discussed in Section 1.6.2. This chapter examines non-specific causes of suppression of one bacterium by another. As defined in Chapter 1, in this thesis "specific" factors refers to inhibitors such as bacteriocins while "non-specific" factors refer to those that cause inhibition by mechanisms other than bacteriocins. Faster growing bacteria are often observed to cause suppression of slower bacteria and this is known as the "Jameson Effect" (Stephens *et al.*, 1997). The potential consequences of this have been explored in two simulation studies by Coleman *et al.* (1996) and (2003). Coleman *et al.* (1996) considered the suppression of *S. typhimurium* by indigenous bacteria of the colon. The prevention of symptoms when healthy subjects were fed $5x10^7$ *S. typhimurium* microbes may have been the result of the competition of indigenous microflora of the gastrointestinal tract. Coleman *et al.* (2003) developed a simulation study to explore the impact of microbial ecology of meat and poultry products on predictions from exposure assessment scenarios. Unconstrained exponential growth models (i.e. those that ignored the effect of other bacteria on the potential for pathogen growth) overestimated *L. monocytogenes* growth as the indigenous microbes on the chicken meat studied were ignored. Coleman *et al.* (2003) related this observation to the "Jameson Effect". The reason for suppression of other bacteria by the faster growing bacteria may be due to production of inhibitory compounds/chemicals including hydrogen ions (pH), bacteriocins, organic acid end products, chemical messengers such as acyl homoserine lactones involved in quorum sensing (Han *et al.*, 2002) and/or other end products of metabolism. Alternatively the 'dominant' bacteria may deplete the media of nutrients or substances required by the other bacteria for growth.

In this Chapter the effect of pH, nutrients and end products of metabolism are examined to determine their role in growth suppression of *L. monocytogenes*. Growth experiments are conducted in 'spent' broth, i.e. broth in which another culture

has been grown to stationary phase and then removed, as this provides a cell-free system that is nutrient depleted, low in pH and contains the end products of bacterial metabolism. These properties, with the exception of metabolic by-products, are easily restored to pre-culture levels. Thus multifactorial experiments can be conducted to determine which of those factors, if any, are responsible for Maximum Population Density (MPD) suppression of *L. monocytogenes* in monoculture. 'Spent' broths from axenic cultures of a variety of foodborne bacteria including *Escherichia coli* (M23), *Pseudomonas fluorescens* (44), *Leuconostoc mesenteroides* and *Lactobacillus sakei* are used to determine whether any effects observed on *L. monocytogenes* growth potential are species specific. Spent broths were characterised prior to use in *L. monocytogenes* inoculation studies by assessment of some or all of the following: glucose concentration, lactate concentration, pH and MPD reached by the parent culture.

The concept of biological control of *L. monocytogenes* was introduced in Chapter 3. The ideal bio-control microorganism on ready-to-eat meat would not decrease product quality. Neither *E. coli* nor *P. fluorescens* satisfy this criterion, and are thus not recommended as bio-control bacteria against *L. monocytogenes* but are included as organisms not normally considered to be inhibitory to *L. monocytogenes* growth. *L. monocytogenes*, *P. fluorescens* and *E. coli* were selected for study as they do not produce bacteriocins and so that the data generated will be comparable to that of Davidson (*unpubl*). Lactic acid bacteria (LAB) were of greater interest in this chapter as bio-control bacteria against *L. monocytogenes* as they are naturally occurring bacteria on ready-to-eat meats and often do not cause overt spoilage, especially homofermentative species. *Le. mesenteroides* and *Lb. sakei* are common LAB found on meat (*see* Chapter 3).

4.2 Material and Methods

4.2.1 Bacterial strains

The five bacteria used in 'spent' broth studies were *L. monocytogenes* (Scott A), *Escherichia coli* (M23), *Pseudomonas fluorescens* (44), *Le. mesenteroides* and *La. sakei* (*see* Appendix 1). Neither *E. coli*, *P. fluorescens* or the LAB (see Chapter 3) produce bacteriocins against *L. monocytogenes* (Scott A).

4.2.2 Inoculum preparation

4.2.2.1 Inocula for generation of 'spent' broths

A stationary phase inoculum of each strain was prepared as follows. Five colonies from a plate were touched with a sterile loop and inoculated into 10 mLof pre-warmed TSB-Ye in a 30 mL McCartney bottle. The broth was incubated without shaking at 25°C for 24 h. 10mL of MRS broth was used to produce a primary culture of the two LAB strains. Cultures were serially diluted in 0.% peptone water (PW) as required.

4.2.2.2 L. monocytogenes *and* P. fluorescens *for inoculation into* L. monocytogenes, E. coli *and* L. monocytogenes *'spent' broths.*

A stationary phase *L. monocytogenes* or *P. fluorescens* culture was prepared as described in Section 4.2.2.1. The culture was serially diluted in PW. 100µl of the 10^{-2} dilution was added to 10mL 'spent' broth preparations to achieve an inoculum level of ~10^4 CFU.mL^{-1}. When a lower inoculum level of *L. monocytogenes* was required, 100µL of the 10^{-4} dilution was added to 10mL 'spent' broth preparations to achieve an inoculum level of ~10^2 CFU.mL^{-1}.

4.2.3 Preparation and modification of 'spent' broths

4.2.3.1 Preparation of 'spent' broth

Cultures were prepared as described in 4.2.2.1. 50µL of those cultures was then aseptically dispensed into 50mLs of sterile TSB-Ye medium in a 100mL conical flask and incubated with shaking (~80 oscillations/minute) in a water bath at 25°C

for either 18 or 24 h, depending on the time taken for pH to decline. For experiments requiring a larger volume of 'spent' broth, 100 or 150µL of culture was aseptically dispensed into 100 or 150mLs of sterile TSB-Ye medium in a 250mL conical flask. Cultures were enumerated by Viable Count (VC) prior to harvesting by serial dilution in PW and surface plating of 100μL of appropriate dilutions onto TSB-Ye plates. Plates were incubated for 48 h at 37°C or 25°C for 24h for LAB strains.

Cultures were harvested by centrifugation at 4000x*G* for 15 minutes to pellet the majority of cells. The supernatant from each spent broth was then filter sterilised using 0.22µm filters to remove any remaining cells. The pH for all cell-free spent broths was determined from aseptically sampled 250µL aliquots.

As required, 10mL aliquots of 'spent' broth were aseptically dispensed into sterile 30mL McCartney bottles. The 'spent' broths were either immediately refrigerated (4 to 5 °C) for use the next day or modified as described below in 4.2.3.2 and then refrigerated (4 °C to 5 °C). The purity of the 'spent' broth preparations was checked periodically during the inoculation studies by plating onto non-selective medium (Plate Count Agar). Selective media was used to determine the number of *P. fluorescens*, *E. coli* and LAB namely; CFC agar (Oxoid CM559 with SR103 supplement), EMB (Oxoid CM69) and MRS respectively.

LAB are usually cultured in specialised media, for example MRS, that meet their low pH and high glucose requirements. However, TSB-Ye is used in these 'spent' broth studies to enable comparison to the data of Davidson (*unpubl*). Thus, prior to commencement of the 'spent' broth studies, the suitability of TSB-Ye as a substrate for growth of LAB was investigated and compared to that of *L. monocytogenes*. The pattern of glucose utilisation and lactate production was also determined. Similarly, *P. fluorescens* and *L. monocytogenes* 'spent' broths were also characterised. Due to time constraints, *E. coli* 'spent' broth was not characterised fully, however, data for pH and MPD were obtained from Mellefont (*unpubl.*).

4.2.3.2 Modification of 'spent' broths

Prepared 'spent' broths as described above were modified as follows (summarised in

Table 4.1). All of the modified 'spent' broths were filter sterilised after manipulations were completed, and the pH measured. 10mL aliquots of modified 'spent' broths were aseptically dispensed into sterile 30mL McCartney bottle and immediately refrigerated for use the next day.

pH

The pH of the spent broth was altered to pH 7.0 by addition of small aliquots of NaOH solutions ranging from 1 to 5M, using sterile pipettes. Some treatments required the spent broth to be returned to 'spent' pH, usually acidic, after addition of nutrients. A sterile glass pipette was used to dispense drops of a 32% HCl to lower the pH.

Nutrients

The nutrient level of the 'spent' broths was returned to that of freshly prepared media by addition of TSB-Ye. The amount added was as per manufacturers instructions, i.e. 0.3g of TSB-Ye was added to 10mL 'spent' broths.

Table 4.1 Symbols and terminology for modified cell-free spent broths.

Broth Description	Plot Symbols	Text Abbreviations
pH adjusted to 7 and dehydrated media was added	(■)	S_o +N(pH7)
Dehydrated media added, no pH adjustment	(□)	S_o +N(pH_)
Dehydrated media added, pH adjusted to 'spent' broth pH	(▲)	S_o +N(pH-S)
pH adjusted to 7, no dehydrated media added	(○)	S_o (pH7)
Unmodified 'spent' broth (pH and nutrients not altered)	(◆)	S_o (pH_)
Control-freshly prepared media	(Δ)	Fo

4.2.4 Growth rate determination for *L. monocytogenes*, *P. fluorescens*, *Le. mesenteroides* and *Lb. sakei* in "pristine" broth

Stationary phase cultures of each strain were prepared as described in Section 4.2.2.1. A small volume (1mL to 1.5mL) of the stationary phase inoculum was aseptically dispensed into 30 mL of appropriate liquid media in a 125mL side-arm

flask to achieve a ~5% drop in transmittance (determined at 540nm with an analogue spectrophotometer). At this density the cell concentration was ~10^{7-8} CFU.mL^{-1}. An un-inoculated side-arm flask containing each medium type was used as a blank for spectrophotometric determinations. Each inoculated flask was incubated in a shaking water bath at 30 °C. The turbidity of the test broth was monitored, with measurement times chosen to correspond, approximately, to successive drops in transmittance of ≤5%. %T values were converted to OD values as described earlier (Section 3.2.5.3) but using a 100%T scale). Log OD was plotted against time and estimates for generation time calculated using linear regression (Section 3.2.5.3). pH, glucose and lactate measurements were undertaken periodically as described in 4.2.6, below.

4.2.5 Inoculation and sampling: Growth rate determinations of *L. monocytogenes* or *P. fluorescens* in spent media

100µl of the 10^{-2} dilution of a stationary phase culture of either *L. monocytogenes* or *P. fluorescens* (Section 4.2.2.1). 100µL of the 10^{-4} dilution for *L. monocytogenes*, was added to 10mL 'spent' broth preparations.

Growth of *L. monocytogenes* and *P. fluorescens* in the 'spent' broth systems was monitored by VC. At regular time intervals, 100μl of the broth was removed and serially diluted in PW as required. For *L. monocytogenes*, 100μL aliquots of appropriate dilutions were surface plated onto either Listeria selective agar (Oxoid CM 856 and Oxford selective supplement SR140E) or TSB-Ye (Oxoid CM 129) plates using a spiral plater (Appendix 2). Plates were incubated at 37°C for 24 h and log viable counts plotted against time. For *P. fluorescens,* 100μL aliquots of appropriate dilutions were surface plated onto TSB-Ye plates and incubated at 25°C for 48h. In studies where only selective media were used, cultures were checked for purity by periodically plating samples onto TSB-Ye.

4.2.6 Measurement of pH, glucose and lactate.

Concurrent to viable count determinations, aliquots of inoculated cultures were removed for pH, glucose and lactate measurements as required. 250μL of culture was required for determination of pH, and pH measurements were undertaken at the

time of sampling. A further 250μL was required for glucose and lactate measurements. Samples for glucose and lactate levels were immediately stored at -18 °C. The following day, samples were rapidly thawed for glucose and lactate measurement using a blood glucose meter (YSI 2300 STAT PLUS , Appendix 2). Where estimates were above the maximum detectable range of the glucose meter (> 27.8 mM), samples were diluted 1:3 in distilled water. The pH was also recorded again at each sample time.

4.3 Results

4.3.1 Characterisation of 'spent' broths

4.3.1.1 LAB in TSB-Ye

The growth rate, MPD, glucose uptake, lactate production and changes in pH of *L. monocytogenes* cultures in TSB-Ye and *Le. mesenteroides* and *Lb. sakei* in MRS and TSB-Ye broth over time are presented in Figures 4.1 to 4.3. For *L. monocytogenes* in TSB-Ye, the glucose content of the medium decreases as the population increases in size and is exhausted in <11 h. Conversely, the lactate concentration increases with increasing population size. For *Le. mesenteroides* in TSB-Ye, glucose is rapidly depleted from the medium (~8 h) but lactate concentration remains unchanged. Similarly for *Lb. sakei* in TSB-Ye the lactate concentration remains unchanged, however, glucose depletion occurs even more rapidly (~3.5 h). Both strains had equal or greater glucose depletion in MRS when compared to TSB-Ye. Lactate concentration remains low for both LAB strains regardless of media type. All strains in all broth systems appear to reach stationary phase at a similar time, between 8 and 10 h.

Estimates of generation time, lag time, glucose content and lactate values are summarised in Table 4.2. While glucose and lactate content were measured for the duration of the experiment, for comparison the values measured at 9.5 h only are presented because all 3 strains had reached MPD by this time in all broth systems trialled.

Table 4.2 Effect of media type on growth kinetics of *L. monocytogenes*, *Le. mesenteroides* and *Lb. sakei* at 30°C and on broth composition after 9.5 h incubation.

Broth	Strain	Broth conditions after 9.5 h incubation			Generation time (h)	Lag time (h)
		pH	%glucose left in broth	Lactate (mM)		
TSB-Ye	*L. monocytogenes*	5.7	ND	15.10	0.98	0.8
	Le. mesenteroides	5.4	ND	0.2	1.36	1
	Lb. sakei	5.5	ND	2.22	1.36	0.5
MRS	*L. monocytogenes*	5.8	99	ND	Insufficient data	2.6
	Le. mesenteroides	5.0	58	ND	1.62	1
	Lb. sakei	4.7	66	ND	1.48	0

ND=not detected

From Table 4.2 it is apparent that while the two LAB strains grow more slowly than *L. monocytogenes* at 30°C in TSB-Ye, the generation time for those strains is relatively unaffected by broth type. *Le. mesenteroides* has a generation time of 1.36 h and 1.62 h respectively in TSB-Ye and MRS. For *Lb. sakei*, a generation time of 1.36 h is observed in TSB-Ye and 1.48 h in MRS. Lag times were similar for all strains in both broth systems. pH, glucose depletion and lactate production were dependent on the broth system used. The pH response and level of glucose depletion after 9.5 h incubation was similar for all strains in TSB-Ye. The lactate level was, however, different with the LAB producing less lactate than *L. monocytogenes*. The level of glucose depletion by all strains is similar in TSB-Ye broth, with all glucose depleted within 9.5 h. However, for LAB grown in MRS significantly more glucose is available and the same or greater quantity of glucose is utilised than TSB-Ye but, apparently, no lactate is produced. It is evident that *L. monocytogenes* grows poorly in MRS and, therefore, this medium was not used in further studies.

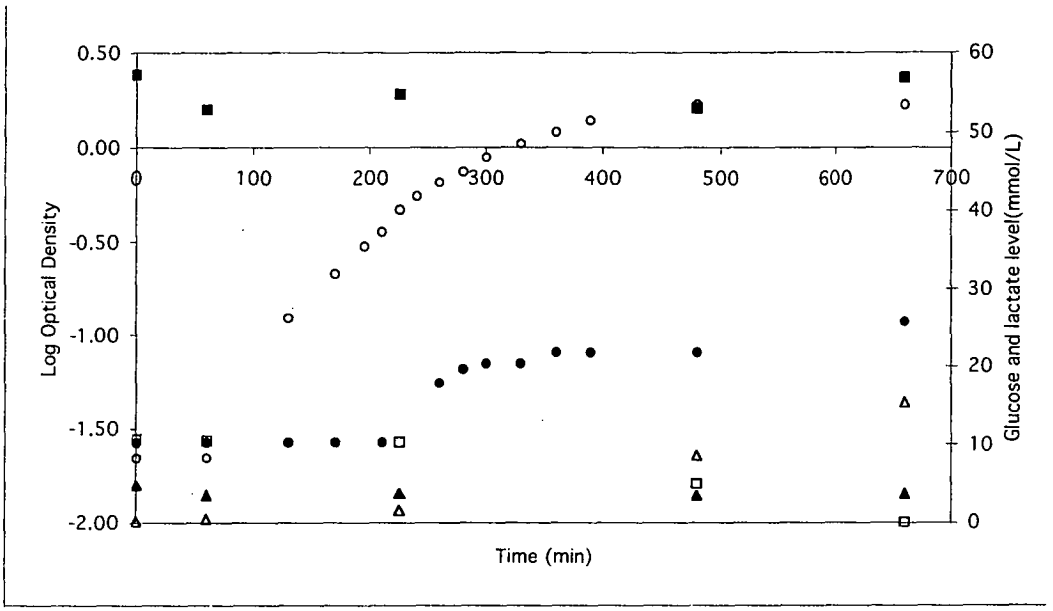


Figure 4.1 Growth, glucose and lactate level of *L. monocytogenes* in TSB-Ye (open symbols) and MRS (closed symbols) broth at 30°C where: (● ○) = growth measured by absorbance, (■/□) glucose (mM) and (Δ/▲) = lactate (mM).

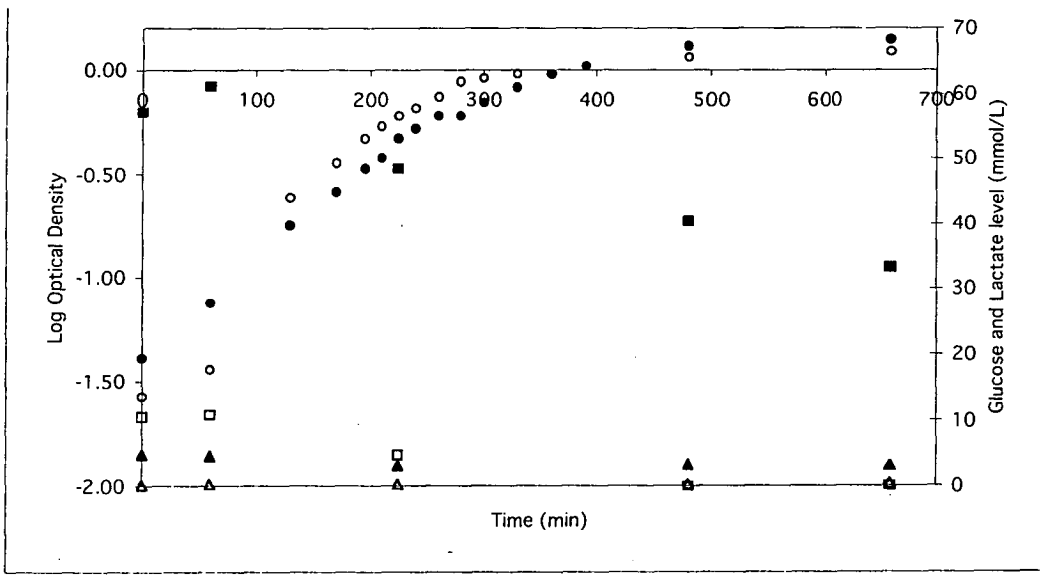


Figure 4.2 Growth, glucose and lactate level of *Le. mesenteroides* in TSB-Ye (open symbols) and MRS (closed symbols) broth at 30°C where: (○/●) = growth measured by absorbance, (□/■) glucose (mM) and (Δ /▲) = lactate (mM).

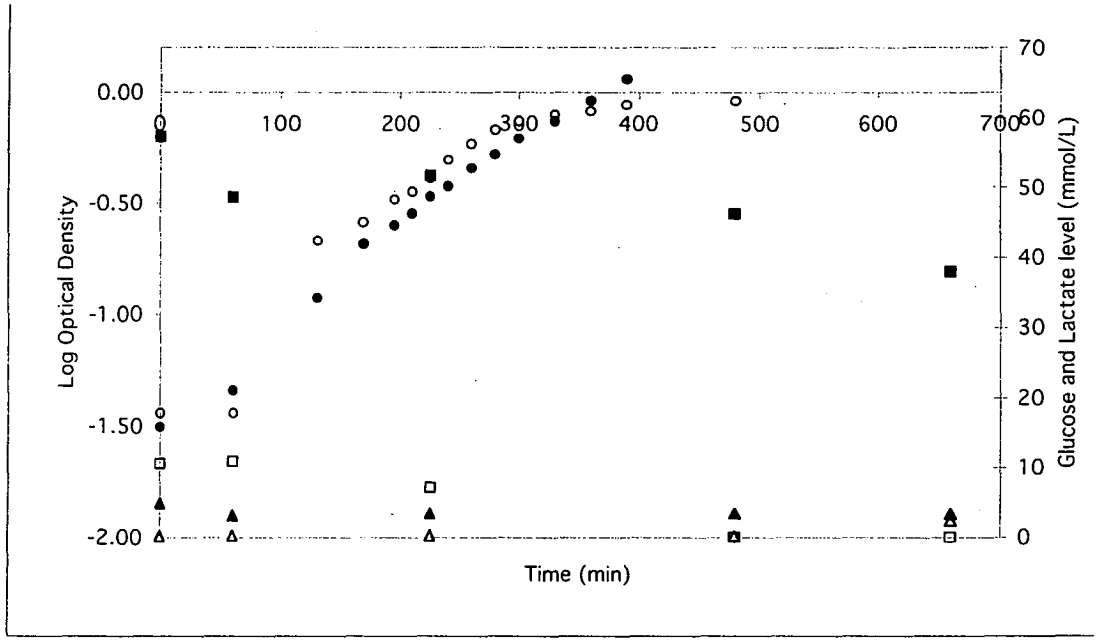


Figure 4.3 Growth, glucose and lactate level of *Lb. sakei* in TSB-Ye (open symbols) and MRS (closed symbols) broth at 30°C where: (●/○) = growth measured by absorbance, (■/□) glucose (mM) and (Δ/▲) = lactate (mM).

4.3.1.2 P. fluorescens, L. monocytogenes *and* E. coli *at 25°C*

pH, glucose and lactate values for *P. fluorescens* and *L. monocytogenes* cultured in TSB-Ye at 25°C are presented in Figures 4.4 to 4.6. For *L. monocytogenes*, the glucose level more than halved after 19.5 to 20.0 h and was depleted by 24 h (n.b. initial glucose level in TSB-Ye is 10-11 mM). pH and log(lactate) were plotted on the same graph to observe the effect on the pH when lactate levels increased. From these results, an incubation time was determined to produce *L. monocytogenes* spent broth with a low pH, *viz.* 18 h. Nonetheless, this broth is only 'partially spent' broth because metabolisable glucose remains, thus this term was adopted for spent broths grown over 18 h. TSB-Ye *L. monocytogenes* broth cultures incubated for 24 h contained no residual glucose, thus they continue to be referred to as 'spent' broth.

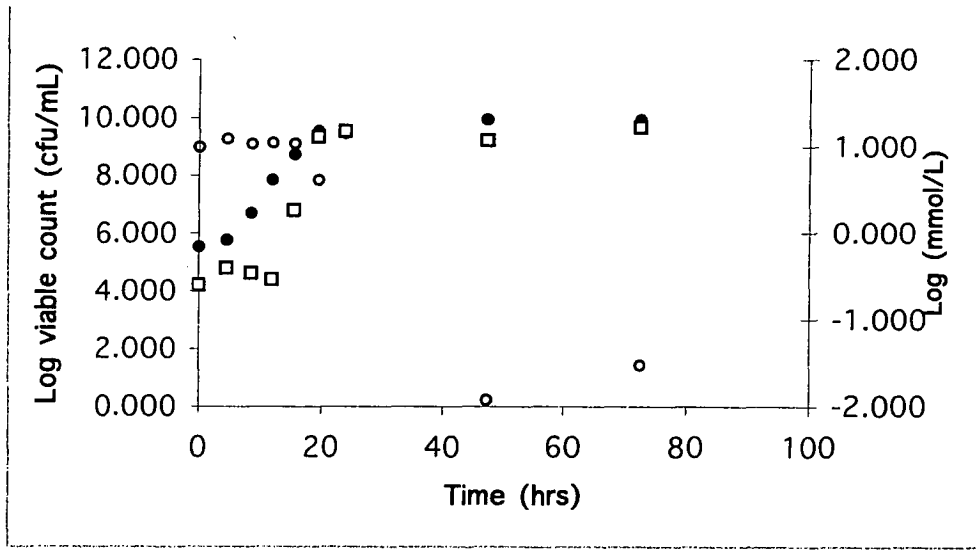


Figure 4.4 Glucose and lactate levels of *L. monocytogenes* grown in "Fresh TSB-Ye" broth at 25 °C (control) where (●) is growth of *L. monocytogenes* (log viable count ($CFU.mL^{-1}$)), (○) glucose) level (log mM) and (□) lactate level (log mM).

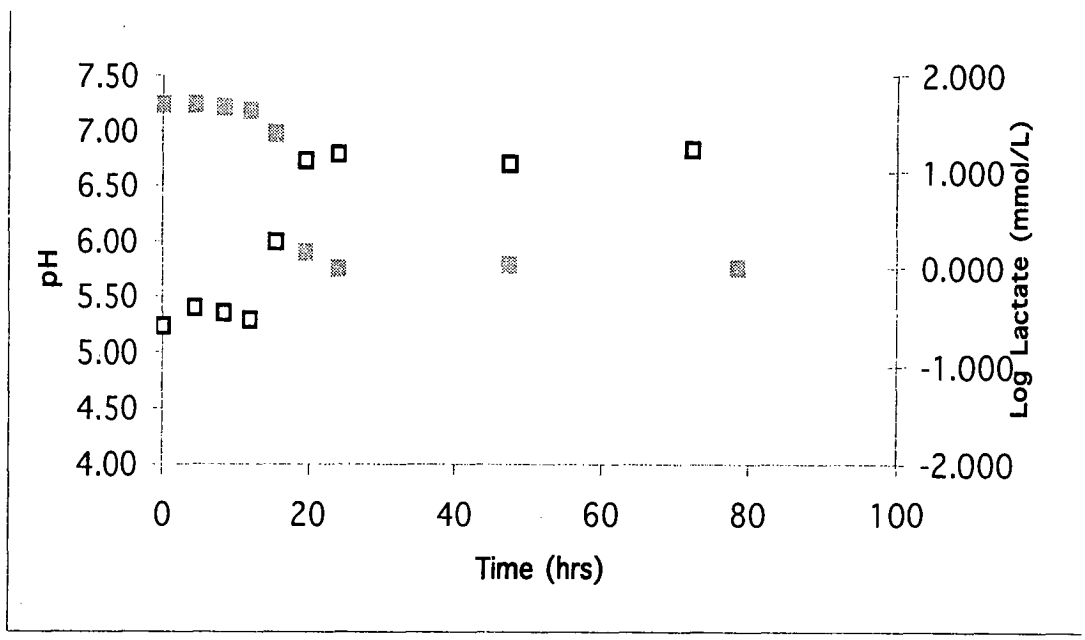


Figure 4.5 pH (▩) and log (lactate) (□) levels in TSB-Ye broth during *L. monocytogenes* growth at 25 °C.

The replicated data for *P. fluorescens* grown in TSB-Ye broth was averaged and is plotted in Fig. 4.7. A temperature of 25°C was used as this was considered near the optimum temperature for growth of *P. fluorescens*.

For *P. fluorescens*, glucose was consumed at a slower rate than other bacteria as seen in Table 4.3. All glucose was consumed by LAB at 7-8 h while 5 mM and 6.38 mM of glucose remained for *L. monocytogenes* and *P. fluorescens* respectively. The pH remained neutral initially and increased after 10 h when cell numbers approached 10^8 CFU.ml^{-1}. No lactate production was detected.

Table 4.3 Glucose level (mM) of different bacteria at various times when cultured in TSB-Ye at 30°C.

Bacterium	0h	1h	3-4h	7-8h	9.5h
L. monocytogenes	10.6	10.5	10.3	5.0	ND
Le. mesenteroides	10.6	10.9	4.8	ND	ND
Lb. sakei	10.6	10.9	7.2	ND	ND
P. fluorescens	10.4	11.0	11.15	6.38	0.50

ND=Not detected

Glucose uptake data was not collected for *E. coli* growth in TSB-Ye due to time constraints. Data for *E. coli* from Mellefont (*unpubl.*) are presented in Figure 4.8 for pH comparisons with other bacteria studied in this Chapter. At 18 h *E. coli* has reached MPD, ~4x10^9 CFU.mL^{-1}, and the pH is at its lowest value, 6.03.

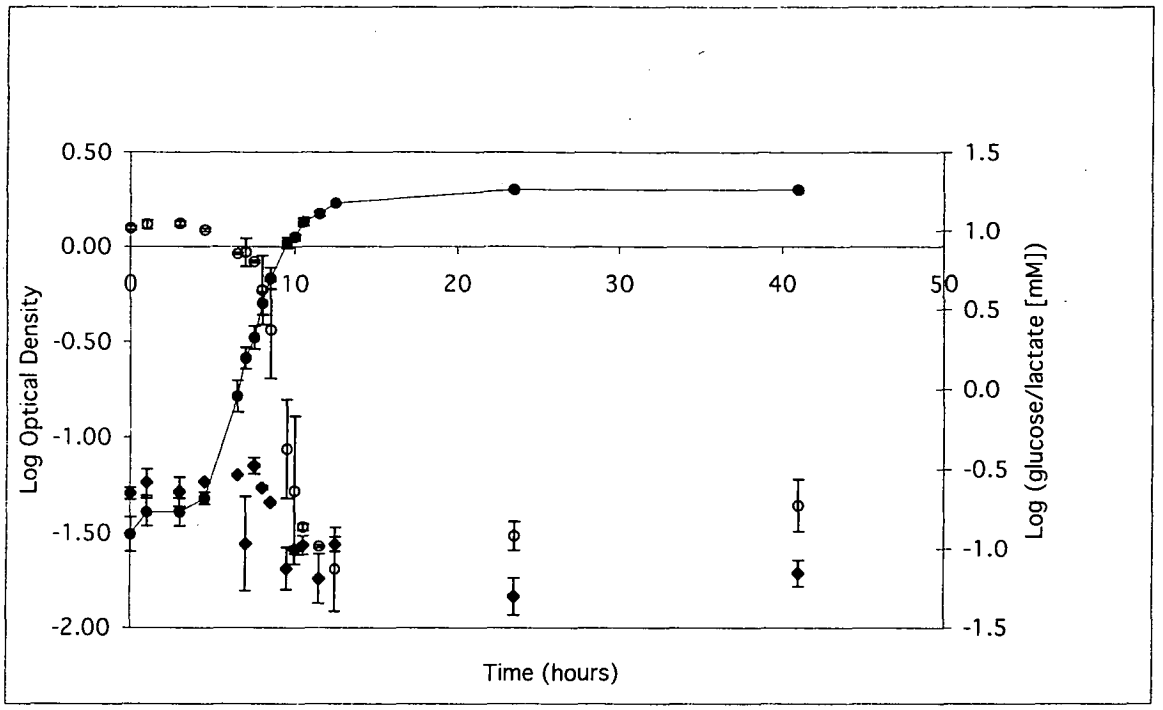


Figure 4.6 Growth, glucose and lactate level of replicate cultures of *P. fluorescens* in TSB-Ye broth at 25°C where: (●) = growth measured by absorbance, (○ glucose mM) and (◆) = lactate (mM).

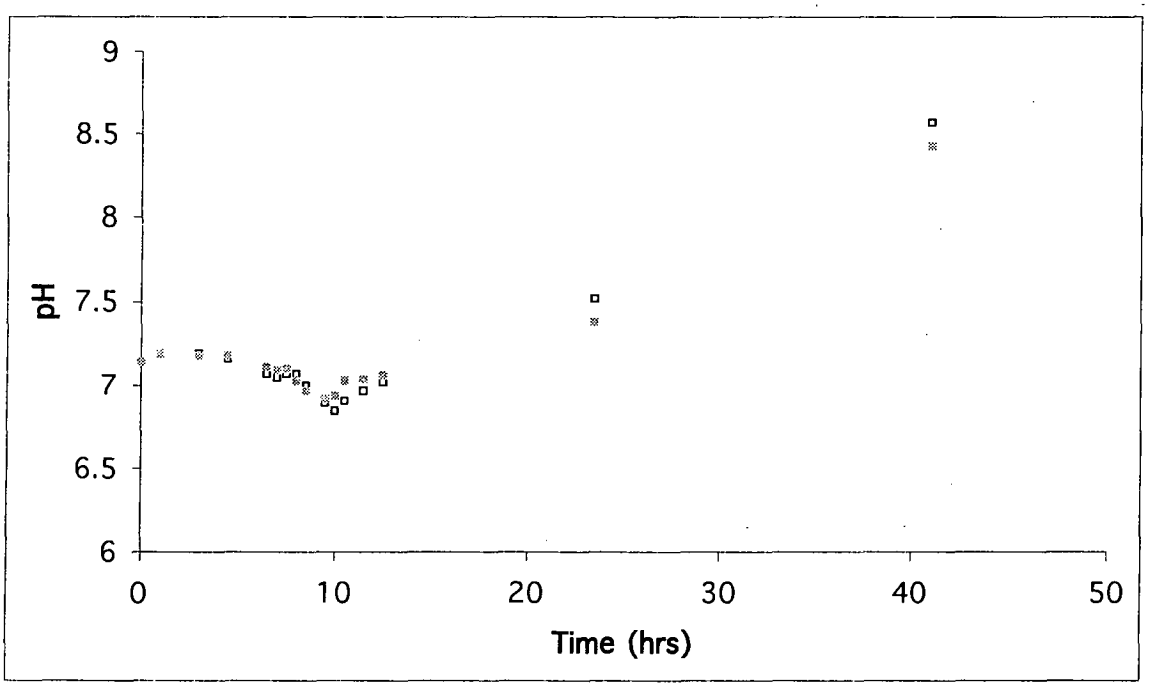


Figure 4.7 pH changes in TSB-Ye broth during growth of *P. fluorescens* at 25 °C (□ : rep 1; ▨: is rep 2).

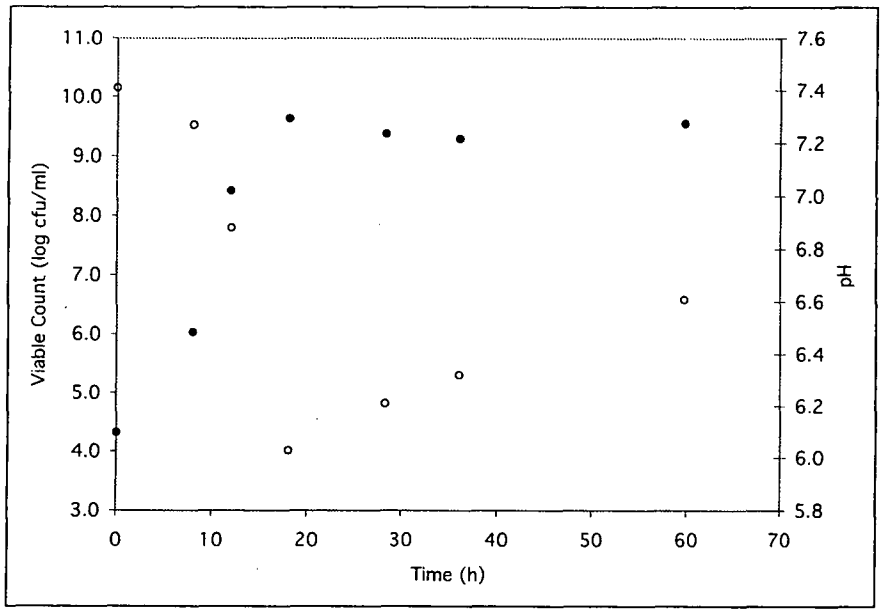


Figure 4.8 Growth (●) and pH (○) of *E. coli* cultured in TSB-Ye at 25°C (data of Mellefont, *unpubl.*).

4.3.2 Kinetics of *L. monocytogenes* in 'spent' broths

For ease of reference, an overview of the studies undertaken in the 'spent' broth systems is presented in Table 4.4. In that summary the details of the pH of the system prior to re-inoculation are included, i.e. the pH of the 'spent' broth preparations, as well as the pH of the systems after they had been modified with either nutrients and/or pH, and a system of abbreviations for the various treatments is introduced

4.3.2.1 Kinetics in 'spent' broths (24 h preparations)

The growth of *L. monocytogenes* in 'spent' broth, generated from *P. fluorescens*, *E. coli* and *L. monocytogenes* are presented in Fig. 4.9 to Fig. 4.11.

Table 4.4 An overview of experiments conducted assess the growth of *L. monocytogenes* in 'spent' broth systems

Experiment	Strain inoculated	Spent Broth generated from:	'Spent' broth type	Figure	pH of spent broth, before adjustment	Symbols for manipulations of spent broth Ψ	Abbreviations in Text	pH after manipulations
1.1	*L. monocytogenes*	*L. monocytogenes*	24 h-full	4.9 (a and b)	6.39	(■) (□) (○) (◆)	So+N(pH7) So+N(pH_) So(pH7) So(pH_)	7.19 6.80 7.27 6.39
1.2	*L. monocytogenes*	*E. coli*	24 h-full	4.10 (a and b)	6.65	(■) (□) (◆)	So+N(pH7) So+N(pH_) So(pH_)	7.21 6.89 6.65
1.3	*L. monocytogenes*	*P. fluorescens*	24 h-full	4.11 (a and b)	7.22	(■) (□) (○) (◆)	So+N(pH7) So+N(pH_) So(pH7) So(pH_)	7.22 7.34 7.22 7.22
2.1	*P. fluorescens*	*L. monocytogenes*	18 h-partial	4.27 (a and b)	5.39	(■) (□) (○) (◆)	So+N(pH7) So+N(pH_) So(pH7) So(pH_)	6.99 6.43 7.00 5.39
2.2	*L. monocytogenes*	*L. monocytogenes*	18 h-partial	4.12 (a and b)	5.39	(■) (□) (◆)	So+N(pH7) So+N(pH_) So(pH_)	7.15 6.43 5.39
3.1	*L. monocytogenes*	*L. monocytogenes*	18 h-partial	4.3.4.13 (a and b)	5.54	(▲) (○) (◆)	So+N(pH-S) So(pH7) So(pH_)	5.84 7.11 5.54
4.1	*L. monocytogenes*	*L. monocytogenes*	18 h-partial	4.14 (a and b)	5.36	(■) (▲) (○) (◆) (Δ)	So+N(pH7) So+N(pH-S) So(pH7) So(pH_) Fo	7.20 6.39 7.49 5.36

Experiment	Strain inoculated	Spent Broth generated from:	'Spent' broth type	Figure	pH of spent broth, before adjustment	Symbols for manipulations of spent broth $^{\Psi}$	Abbreviations in Text	pH after manipulations
4.1	*L. monocytogenes*	*L. monocytogenes*	18 h-partial	4.3.14 (a and b)	5.36	(■) (▲) (○) (◆) (Δ)	So+N(pH7) So+N(pH-S) So(pH7) So(pH_) Fo	7.20 6.39 7.49 5.36
4.2	*L. monocytogenes* **10^2 CFU.mL^{-1}	*L. monocytogenes*	18 h-partial	4.3.15 (a and b)	5.36	(▲) (◆)	So+N(pH-S) So(pH_)	5.57 5.36
4.3	*L. monocytogenes*	*E. coli*	18 h-partial	4.3.4.16 (a and b)	6.39	(■) (▲) (○) (◆) (Δ)	So+N(pH7) So+N(pH-S) So(pH7) So(pH_) Fo	7.06 6.39 7.40 6.39 7.27
5.1	*L. monocytogenes*	*Le. mesenteroides*	18 h-partial	4.3.4.17 (a and b)	5.95	(■) (▲) (○) (◆) (Δ)	So+N(pH7) So+N(pH-S) So(pH7) So(pH_) Fo	7.09 5.94 7.0 5.94 7.24
5.2	*L. monocytogenes*	*Lb. sakei*	18 h-partial	4.3.4.22 (a and b)	5.96	(■) (▲) (○) (◆) (Δ)	So+N(pH7) So+N(pH-S) So(pH7) So(pH_) Fo	7.05 6.02 7.16 5.97 7.24

(■) The pH was adjusted to 7 and new dehydrated media added (3%TSB and 0.6% YE).
(□) New dehydrated media added (3%TSB and 0.6% YE), no pH adjustment .
(▲) New dehydrated media added (3%TSB and 0.6% YE), pH adjusted to spent pH.
(○) The pH was adjusted to 7, no dehydrated media added.
(◆) The pH remained unaltered and no dehydrated media is added – i.e. unmodified 'spent' broth
(Δ) Control- Fresh dehydrated media (3%TSB and 0.6% Ye).
So 'spent' broth
Fo Fresh broth/control
N Nutrients (i.e. addition of 3%TSB and 0.6% YE
(pH) denotes pH of spent broth manipulations where "7" is neutral and "_" denotes no amendment

The treatments with added nutrient appeared to achieve a MPD 2 to 3 logs greater than spent broth and spent neutral broth. The MPD for nutrient supplied and neutral pH/nutrient added was similar, whereas the MPD for *L. monocytogenes* grown in *P. fluorescens* spent broth was the same for all four treatments. The initial pH for all treatments was around 6-7. When *L. monocytogenes* was grown in its own 'spent' broth a drop in pH was noted for nutrient added treatments whereas the pH of the 'spent' broth (Fig. 4.9, 4.10) remained the same and the 'spent' neutral broths experienced a slight drop in pH.

The MPD of *L. monocytogenes* cultured in its own, and *E. coli*, spent broth with TSB-Ye added, was ~10^9 CFU.mL^{-1} compared to the lower MPD (~10^7 CFU.mL^{-1}) upon growth in unamended 'spent' broth and 'spent' neutralised broth alone (Figure 4.9 and 4.10). All pH changes occurred after ~20 h of incubation.

L. monocytogenes spent broth

After 20-25 h S_o+N(pH_) reached MPD and S_o+N(pH7) reached MPD 5 h later (Figure 4.9a). The pH was close to neutral for both broths thus no conclusions were possible regarding pH effect. pH decreased as MPD was reached (Figs. 4.9a and b). Almost 5 logs of growth occurred in the nutrient added broths however the pH only declined after 20 h when most of the glucose substrate nutrient was utilised. 'Spent' broth, S_o (pH6.39), supported limited *L. monocytogenes* growth, but no pH change (Fig. 4.9b) occurred, suggesting that glucose has been depleted from the 'spent' broths. However, pH-neutral broth with no added nutrient, S_o (pH7), also supported growth but the pH increased for the first 10 h showing that substrates leading to basic end products of metabolism (e.g. amino acids or peptides) were utilised.

E. coli spent broth:

L. monocytogenes reached MPD within 20 h in S_o+N(pH7) and S_o+N(pH_) when grown in *E. coli* spent broth. The pH was 7.21 and 6.89 for S_o +N(pH7) and S_o +N(pH_) respectively. The pH was also close to neutral for both nutrient added broths (Fig. 4.10b). pH increase was noted after 20 h incubation for all three treatments. There was insufficient data to calculate the MPD for spent broth.

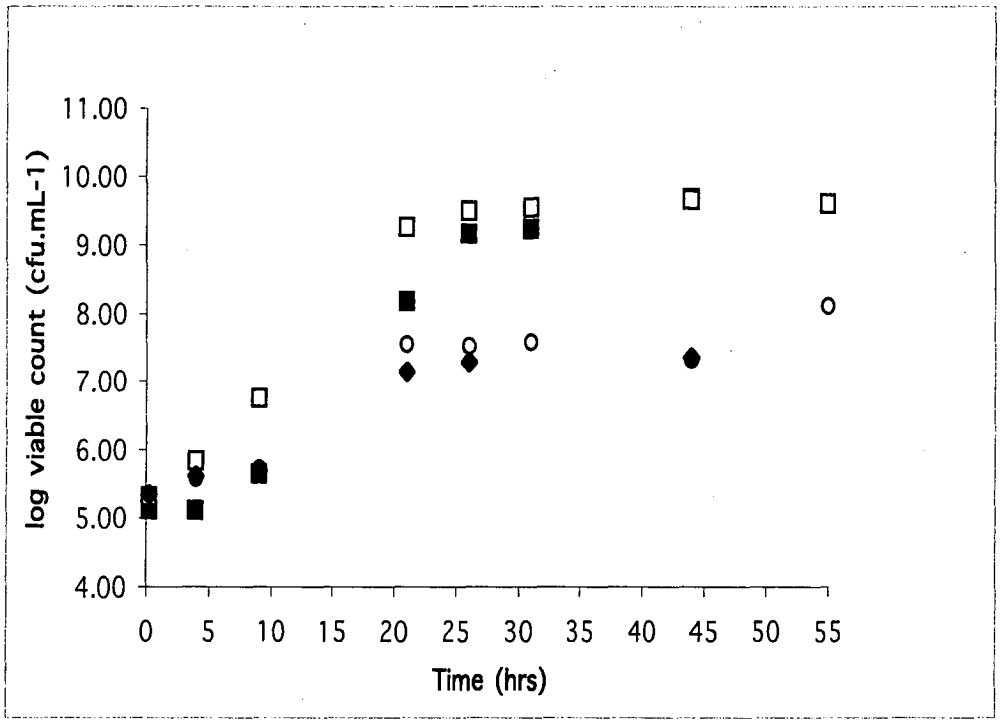


Figure 4.9 a The growth of *L. monocytogenes* in *L. monocytogenes* spent broth where (■) is pH7/TSB-Ye (7.19), (❑) is TSB-Ye/unaltered pH (pH 6.80), (○) pH7 (7.27) and (♦) spent (pH 6.39).

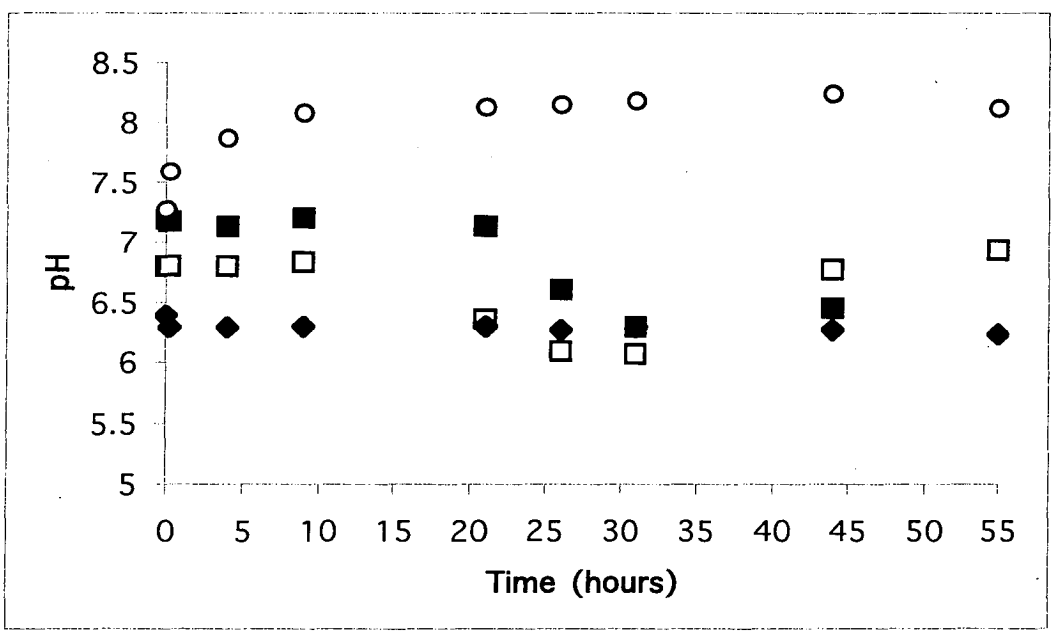


Figure 4.9 b pH changes in broths during growth of *L. monocytogenes* in *L. monocytogenes* 'spent 'broth where (■) is pH7/TSB-Ye, (□) is TSB-Ye/unaltered pH, (○) pH7 and (♦) is 'spent'.

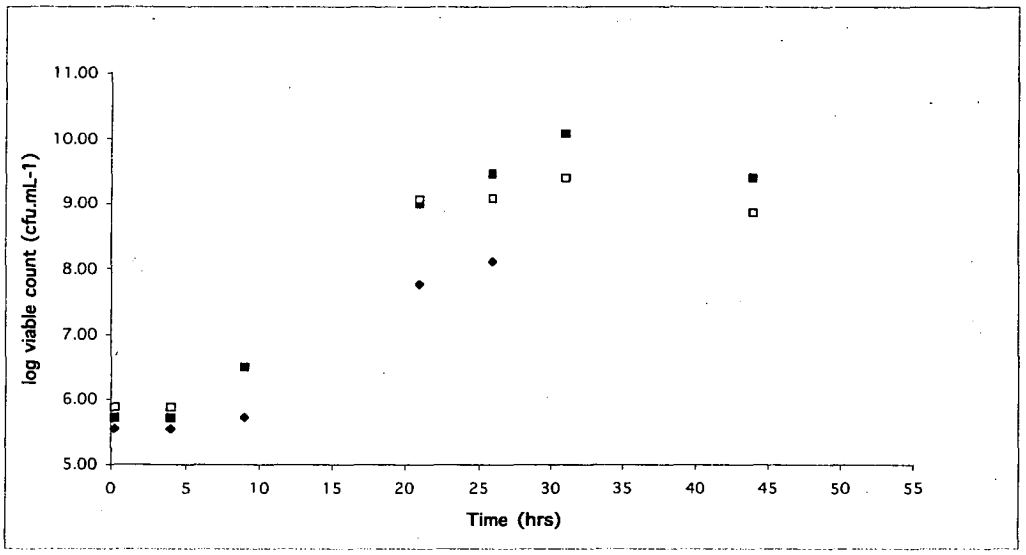


Figure 4.10a Growth of *L. monocytogenes* in *E. coli* in 'spent' broth, where (■) is pH7/TSB-Ye, (□) is TSB-Ye/unaltered pH (pH 6.89) and (♦) 'spent' (pH 6.65).

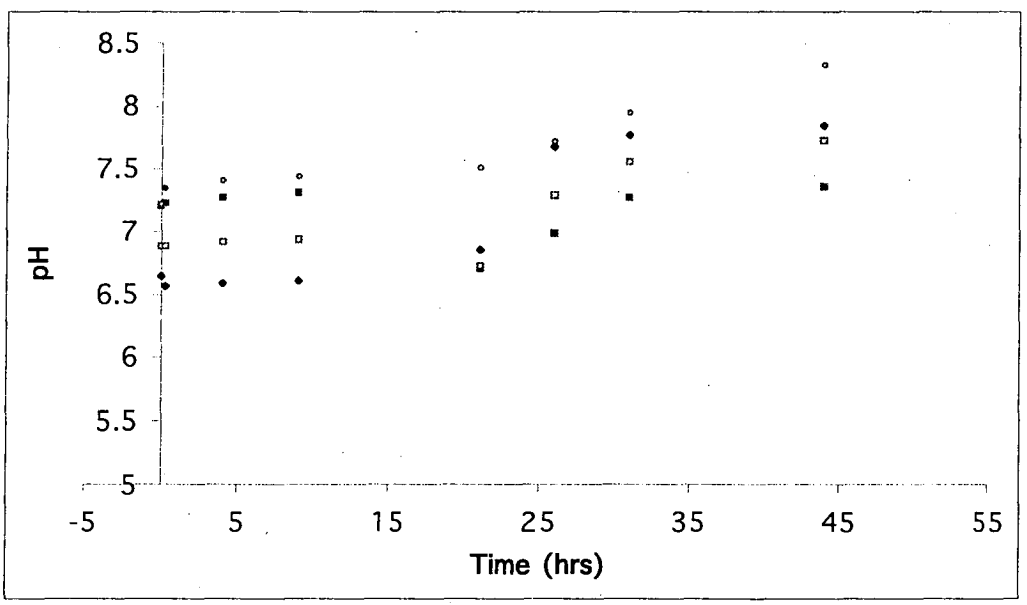


Figure 4.10b pH changes in broth during growth of *L. monocytogenes* in *E. coli* spent broth, where (■) is pH7/TSB-Ye, (□) is TSB-Ye/unaltered pH, (○) pH7 and (♦) spent.

<u>*P. fluorescens* spent broth:</u>

L. monocytogenes reached MPD within 20 h for all four treatments when grown in *P. fluorescens* spent broth (Fig. 4.11 (a) and (b)). There was a slight drop in pH for S_o(pH_) and S_o (pH7) and a greater drop in pH for S_o+N(pH_) and S_o+N(pH_).

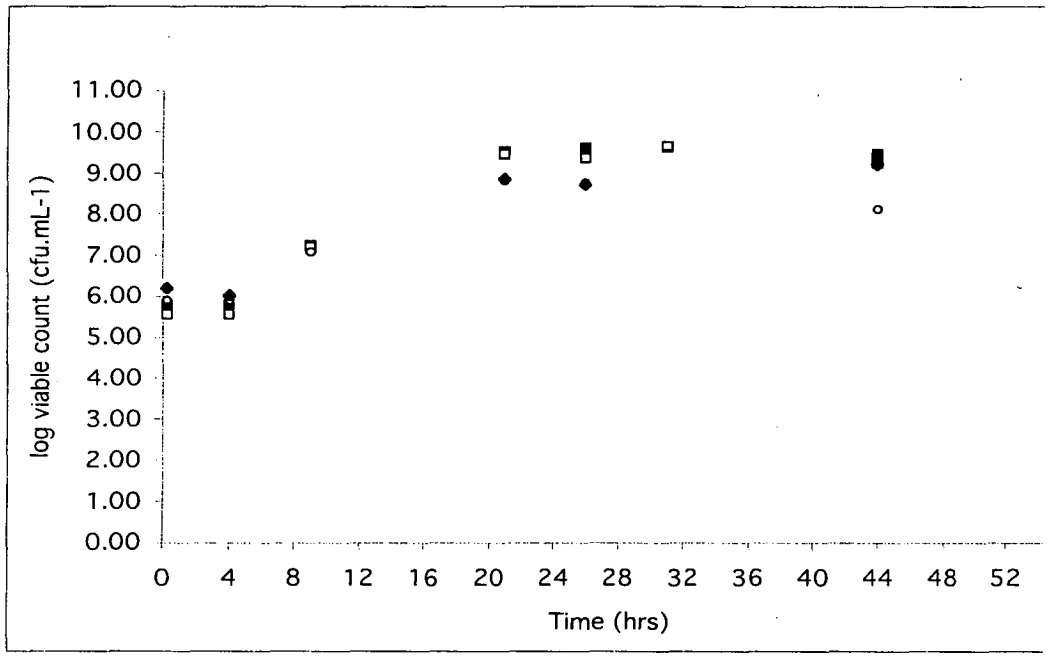


Figure 4.11a Growth of *L. monocytogenes* in *P. fluorescens* 'spent' broth where (■) is pH7/TSB-Ye, (□) is TSB-Ye/unaltered pH (pH 7.35), (○) pH7 and (♦) spent (pH 7.22).

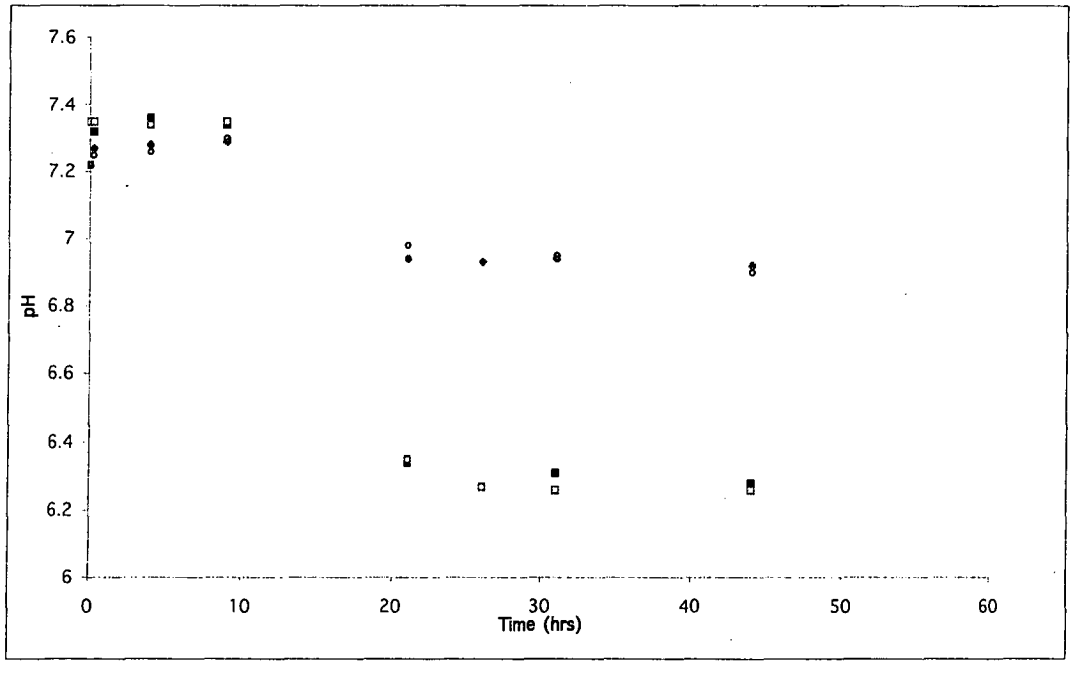


Figure 4.11b pH changes in broth during growth of *L. monocytogenes* in *P. fluorescens* 'spent' broth, where (■) is pH7/TSB-Ye, (□) is TSB-Ye/unaltered pH, (○) pH7 and (♦) 'spent'.

4.3.2.2 Kinetics in 'partially spent' broths

As described earlier, 'partially spent' broths were generated by growth of the appropriate strains in TSB-Ye for 18 h, thus providing a system low in pH but only partially depleted of nutrients.

Growth of *L. monocytogenes* in 'partially spent' broths generated from *L. monocytogenes*

The growth response of *L. monocytogenes* in 'partially spent' broth systems generated by the same strain is presented in Figures (4.12 to 4.19).

The effect of nutrient addition was compared to that of unamended 'partially spent' broth (Figure 4.12 (a) and (b)). Addition of nutrients to a pH-neutral or broth returned to 'pH-spent' system resulted in 4 logs of growth of *L. monocytogenes*. If the pH remained unaltered, i.e. it was acidic, and no nutrients were added, growth occurred at a slower rate but did, however, reached the same MPD as S_o+N(pH7). From the data for the unamended broth it is not clear if MPD was reached by completion of the experiment. Similar to 'spent' broth trials there is a reduction in pH when MPD is reached, and following that the pH increases. 'Partially spent' broth pH did not change throughout the duration of the experiment.

Some of the manipulations described in Figure 4.12 were replicated and the experiment monitored for a longer duration to clarify when *L. monocytogenes* would reach MPD in an unamended 'partially spent' broth (Fig. 4.13). Additionally, a 'partially spent' broth was returned to neutral pH without additional nutrients. The MPD reached by S_o +N(pH-S) broth was ~10^8 CFU.mL^{-1} which was lower than MPD (~10^9 CFU.mL^{-1}) observed in experiments summarised in Figures 4.9 to 4.11. The *L. monocytogenes* growth in the spent and pH7 broths were similar to in experiments summarised in Figure 4.9. However a reduction in pH was evident for S_o+N(pH-S) broth (Figure 4.13b).

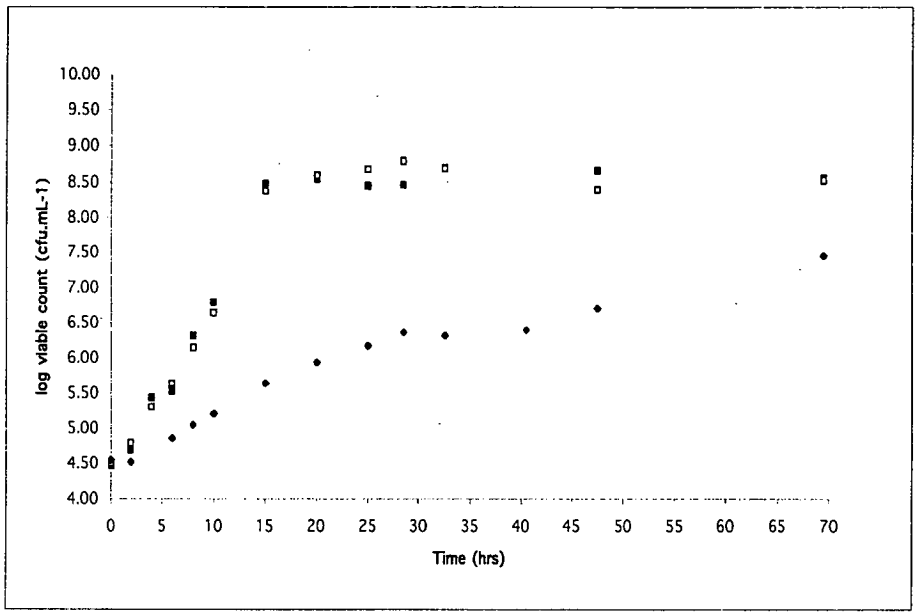


Figure 4.12a. Growth of *L. monocytogenes* in *L. monocytogenes* 'partially spent' broth, where (■) is pH7/TSB-Ye, (□) is TSB-Ye/altered to spent pH (pH 5.39) and (♦) spent (pH 5.39).

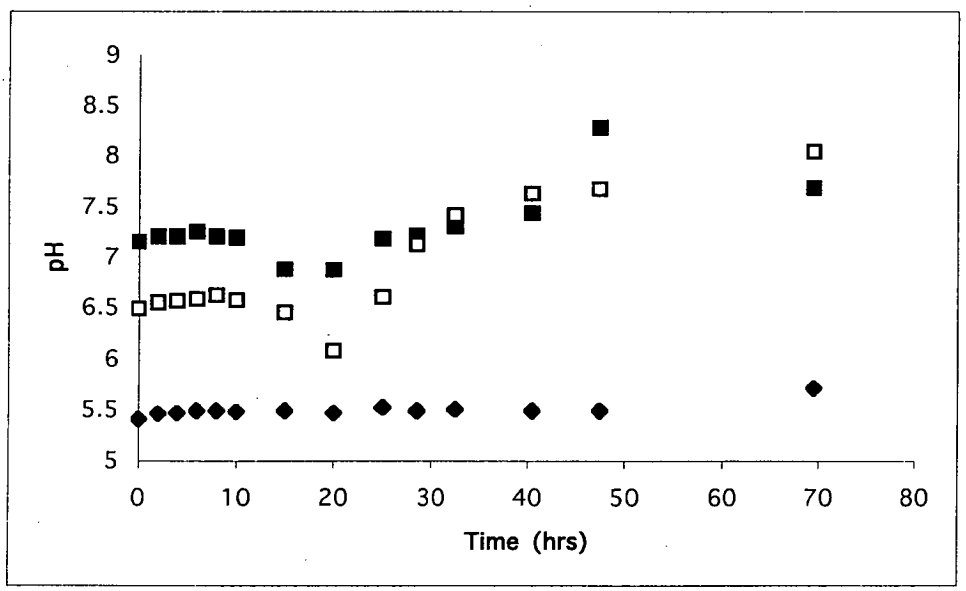


Figure 4.12b. pH changes during growth of *L. monocytogenes* in *L .monocytogenes* 'partially' spent broth, where (■) is pH7/TSB-Ye, (□) is TSB-Ye/unaltered pH and (♦) 'partially spent'

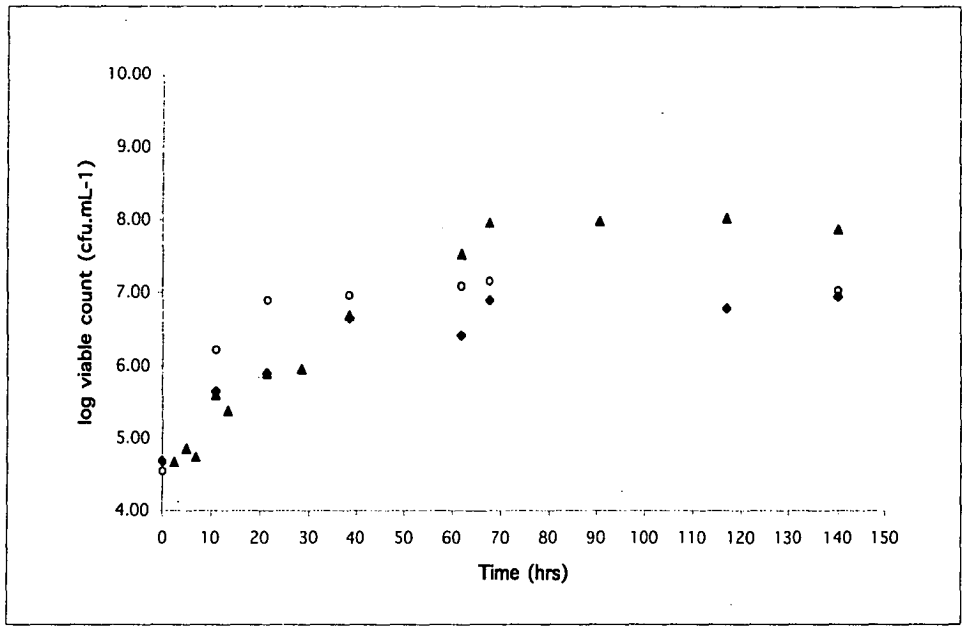


Figure 4.13a Growth of *L. monocytogenes* in *L. monocytogenes* 'partially spent' broth, where (○) is pH7, (▲) is TSB-Ye/ pH altered to spent pH (5.84) and (♦) spent (pH 5.84).

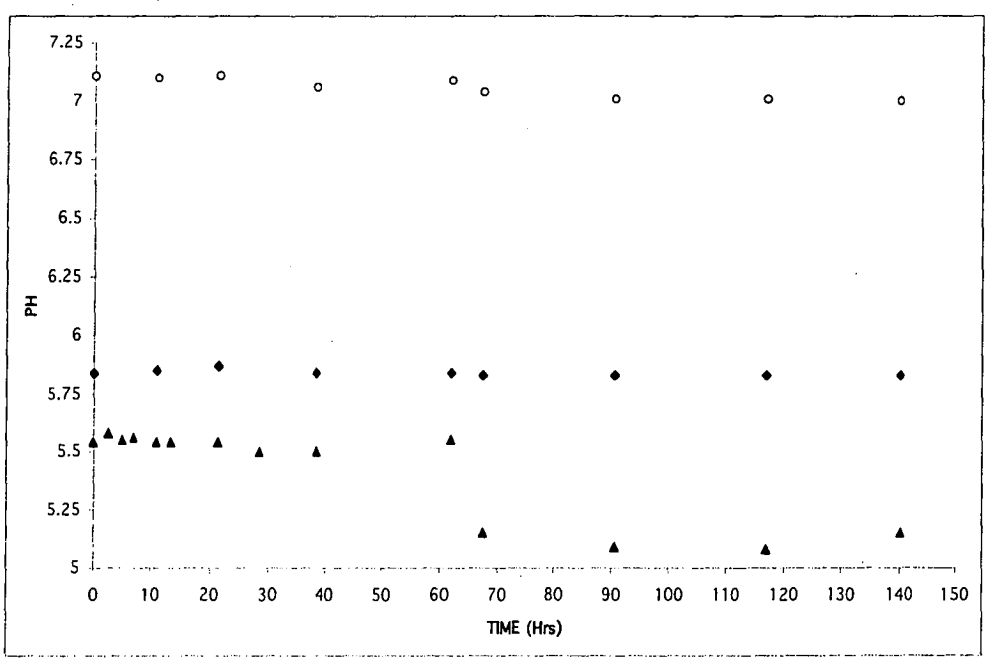


Figure 4.13b pH changes during growth of *L. monocytogenes* in *L. monocytogenes* 'partially spent' broth where (▲) is TSB-Ye/pH altered to spent pH, (○) pH7 and (♦) spent.

To clarify the pattern of responses observed in the earlier studies (refer to data/graphs above), the experiments were repeated and rigorous close-interval sampling of the 'partially spent' broth systems was undertaken. Additionally, a control was included, i.e. *L. monocytogenes* was inoculated into freshly prepared TSB-Ye. The results are presented in Figures 4.14. The MPD reached by *L. monocytogenes* growing in the control broth (Fo) and S_o+N(pH7) is almost the same however S_o+N(pH7) reached as slightly higher MPD. MPD was reached from these broths at about after ~20 h similar to the results in Figure 4.9a. Interestingly, S_o+N(pH-S) reached a MPD similar to the control and S_o+N(pH-7) but at a slower rate, taking approximately 40 h. *L. monocytogenes* grown in 'partially spent' neutral broth showed a slightly higher MPD compared to 'partially spent' broth alone. The MPD reached by the control, S_o+N(pH7) and S_o+N(pH-S), 'partially spent' and pH7 are displayed presented in Table 4.5. The MPD of *L. monocytogenes* grown in nutrient added broths was >9 $\log_{10}$(CFU.mL^{-1}) while spent and pH7 broths were close to 8 $\log_{10}$(CFU.mL^{-1}). The MPD ($\log_{10}$CFU.mL^{-1}) was estimated from the average of the highest points on the graphs for each treatment and are displayed in Table 4.5. MPD was reached by spent and pH 7 broths after 20 h incubation similar to nutrient added broths and the control.

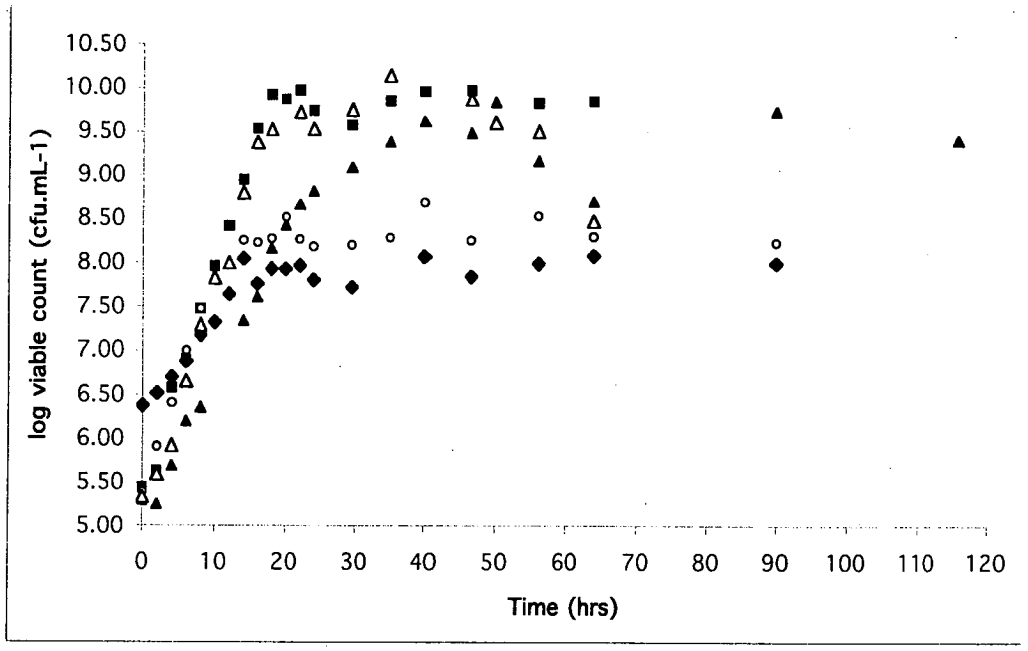


Figure 4.14a Growth of *L. monocytogenes* in *L. monocytogenes* spent broth, where (■) is pH7/TSB-Ye, (▲) is TSB-Ye/altered to spent pH (pH 5.61) (○) is 'partially spent' broth adjusted to pH7 , (♦) 'partially spent' (pH 5.36) and (Δ) control (i.e. new TSB-Ye)

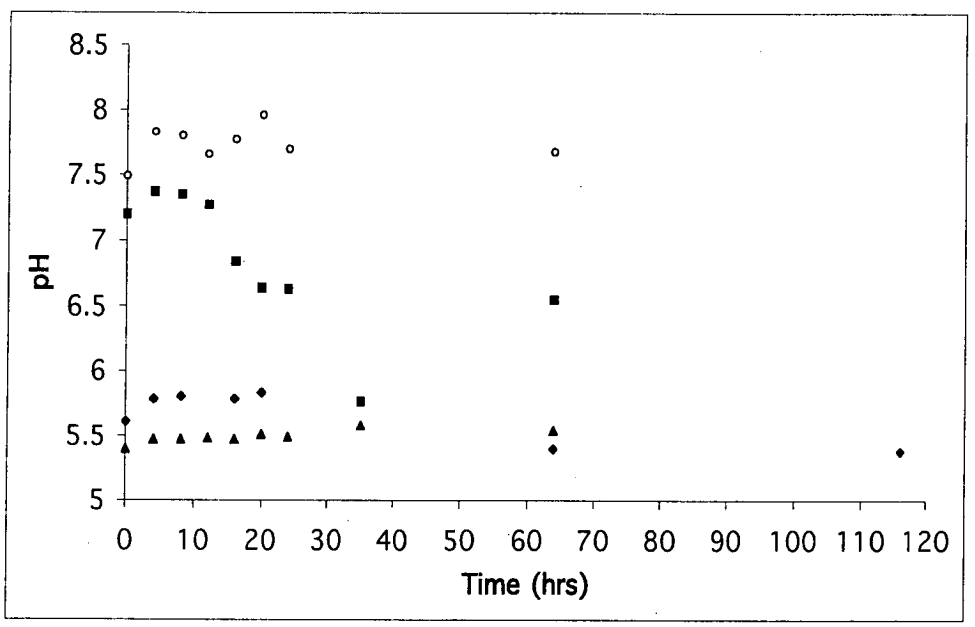


Figure 4.14b The pH of *L. monocytogenes* grown in *L. monocytogenes* 'partially' spent broth where (■) is pH7/TSB-Ye, (▲) is TSB-Ye/altered to spent pH (pH 5.61) (○) is broth adjusted to pH7 and (♦) spent (pH 5.36).

Table 4.5 The MPD (Log viable count ($CFU.mL^{-1}$)) attained by *L. monocytogenes* in *Escherichia coli*,. *L. monocytogenes*, *Le mesenteroides* and *Lb. sakei* 'partially spent' broth over 100 hours at 25 °C.

	Maximum Population Density of *L. monocytogenes* (log $CFU.mL^{-1}$) in spent broth produced from:				
Treatment	*L. monocytogenes*	*L. monocytogenes* -low inoculum	*E. coli*	*Le. mesenteroides*	*Lb. sakei*
PH7 + TSB 3% (yeast 0.6%)	9.91	Not tested	9.78	9.95	9.93
TSB 3% (yeast 0.6%) + pH adjusted to spent	9.56	9.52	9.75	9.73	9.84
PH7	8.52	Not tested	8.91	8.24	8.27
Spent	8.00	8.27	8.81	8.60	8.52
"Fresh" TSB-Ye, control	9.96	9.93	9.93	9.93	9.96
Figure	4.15 (a)	4.15 (a)	4.16a	4.17a	4.22a

Finally, the effect of a low inoculum level on the subsequent growth of *L. monocytogenes* in 'partially spent' broths generated from the same organism was examined. In an unamended 'partially spent' broth, 4 logs of growth was achieved. Addition of nutrients to the pH-spent system allowed a further log of growth, i.e. 5 logs of growth in total. pH remained low (~5.5) in the nutrient amended system. The pH returned to neutral after 70h in the unamended 'partially spent' broth.

The effect of inoculating with a lower inoculum (10^2 - 10^3 $CFU.mL^{-1}$) into *L. monocytogenes* spent broth, in S_o+N(pH_) and S_o(pH_) modified spent broths, is shown in Figure 4.15a and summarised in Table 4.5. The desired starting inoculum number was 10^2 $CFU.mL^{-1}$, however, due to experimental error a higher inoculum resulted, 10^3 $CFU.mL^{-1}$. MPD was attained after ~20 h for *L. monocytogenes* inoculated at $10^{4.5\text{-}5.5}$ $CFU.mL^{-1}$ and within 40 h at the lower inoculum (~$10^{3.5}$ $CFU.mL^{-1}$). However, a similar MPD was reached for both inoculum levels as seen in Table 4.5. On the other hand, a low *L. monocytogenes* inoculum level, affected the growth rate of *L. monocytogenes* in its own spent broth as seen in Table 4.9. The generation time was increased from 1.79 h and 2.70 h for high inoculum to 2.35 h and 3.90 h for lower inoculum for S_o +N(pH-S) and S_o (pH_), respectively.

Growth of *L. monocytogenes* 'partially spent' broths generated from *E. coli*

The MPD reached for the control, S_o+N(pH7) and S_o+N(pH-S) depicted in Figure 4.16 was similar to MPD reached in Figure 4.15a. The estimated MPD for *L. monocytogenes* grown in different treatments of 'partially spent' broth is presented in Table 4.5. These MPD figures were similar to *L. monocytogenes* grown in 'partially spent' *L. monocytogenes* broth. A slight reduction in pH was evident for S_o(pH7) and 'partially spent' broth similar to other experiments using *L. monocytogenes* and *E . coli* spent broths. A similar drop in pH after MPD was attained was noted for nutrient-supplemented 'partially spent' broths and the control compared to other experiments using *L. monocytogenes* and *E. coli* 'partially spent' broths (Figure 4.16b). The MPD was reached for spent and pH7 at a similar time to nutrient-supplemented 'partially spent' broths (Figures 4.9 to 4.16).

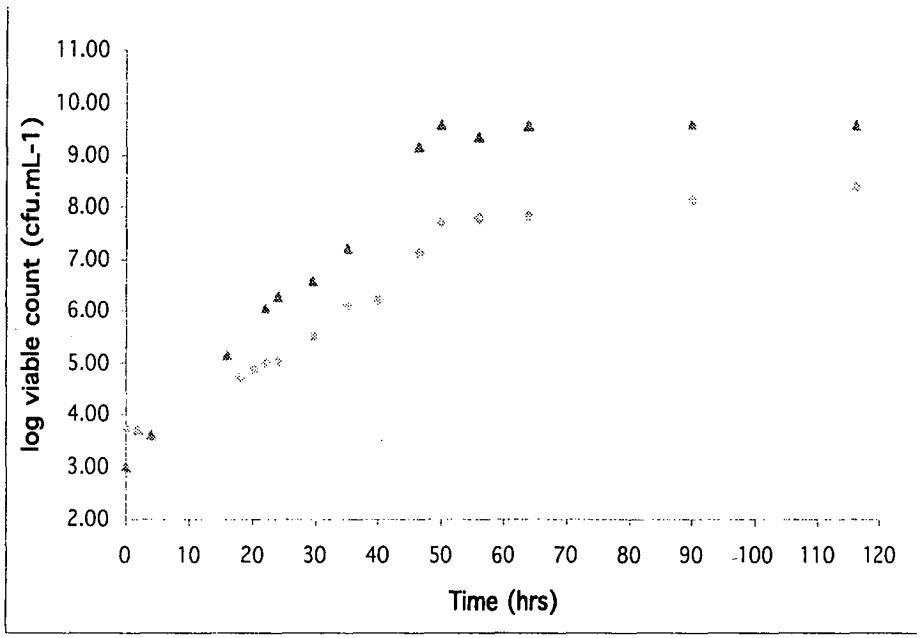


Figure 4.15a Growth of *L. monocytogenes* inoculated at a level of 10^3 CFU.mL^{-1} in *L. monocytogenes* 'partially spent' broth, where (▲) is TSB-Ye/ pH adjusted to spent pH (pH 5.57), (◆) is spent (pH 5.36)

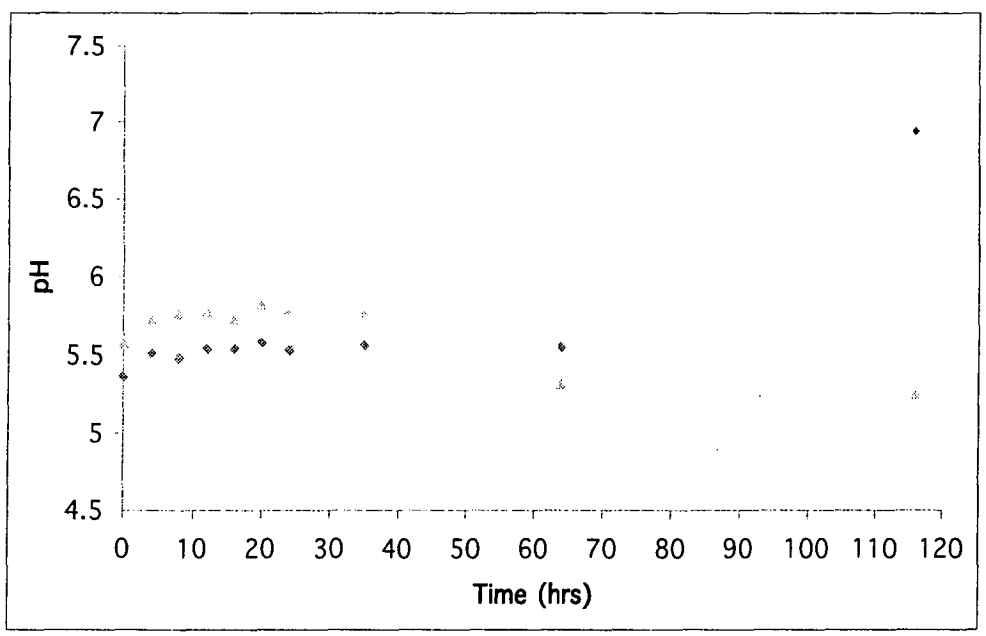


Figure 4.15 b pH changes in *L. monocytogenes* 'partially spent' broth inoculated with *L. monocytogenes* at a level of ~10^3 CFU.mL^{-1}, where (▲) is TSB-Ye/ pH adjusted to 'spent' pH (pH 5.57), (◆) is 'partially spent' (pH 5.36)

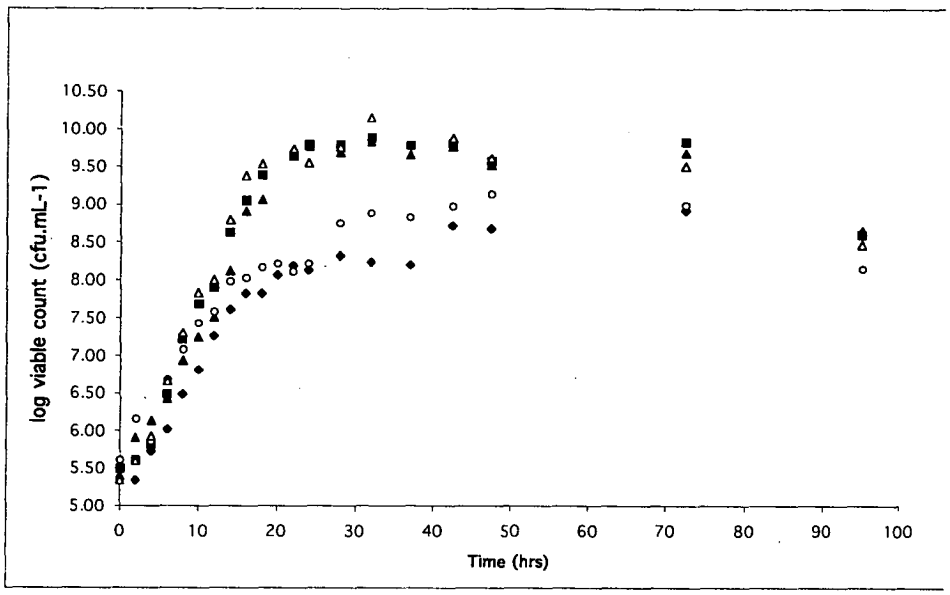


Figure 4.16a Growth of *L. monocytogenes* in *E. coli* 'partially spent' broth, where (■) is pH7/TSB-Ye, (▲) is TSB-Ye/pH altered to 'partially spent' pH(pH 6.39), (○) is broth adjusted to pH7. (♦) is 'partially spent' (pH 6.39) and (Δ) is the control (*L. monocytogenes* is grown in fresh TSB-Ye broth).

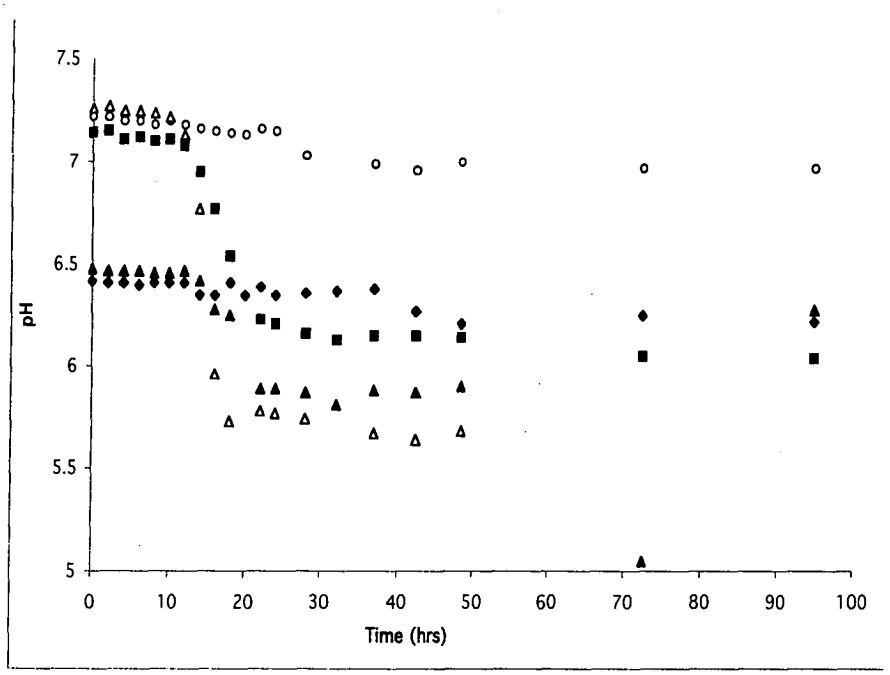


Figure 4.16b pH changes during *L. monocytogenes* growth in *E. coli* 'partially spent' broth over time where (■) is pH7/TSB-Ye, (▲) is TSB-Ye/pH altered to spent pH(pH 6.39), (○) is 'partially spent' broth adjusted to pH7,. (♦) is 'partially spent' (pH 6.39) and (Δ) is the control (*L. monocytogenes* grown in fresh TSB-Ye broth).

Growth of *L. monocytogenes* in 'partially spent' broths generated from *Le. mesenteroides.*

Similar growth trends were observed for *L. monocytogenes* grown in *Le. mesenteroides* 'partially spent' broth or *E. coli* or *L. monocytogenes* 'partially spent' broth as summarised in Table 4.5. A similar reduction in pH was noted for *L. monocytogenes* grown in *Le. mesenteroides* 'partially spent' broth compared to *L. monocytogenes* grown in *L. monocytogenes* and *E. coli* 'partially spent' broth (Figure 4.9 to 4.22).

The glucose and lactate levels of *Le. mesenteroides* 'partially spent' broth after growth of *L. monocytogenes* are shown in Figures 4.18 to 4.21. The initial glucose levels for spent broths after manipulations are shown in Table 4.6 below. The initial glucose level is expected to be 10mM thus errors during addition of supplementary nutrients may have occurred, or low, undetectable amounts of glucose may have been present in the spent broths.

Table 4.6 The initial glucose and lactate levels in modified LAB 'partially spent' broth treatments before *L. monocytogenes* inoculation.

***Le. mesenteroides* spent broth**			***Lb. sakei spent* broth**		
Modifications	*Glucose (mM)*	*Lactate (mM)*	*Modifications*	*Glucose (mM)*	*Lactate (mM)*
TSB-YE/pH7	12.2	0.539	TSB-YE/pH7	13.1	0.475
TSB-YE/pH5.95	12.1	0.473	TSB-YE/pH5.95	13.4	0.459
PH7	ND	0.276	PH7	ND	0.267
Spent	ND	0.318	Spent	0.017	0.282
Fresh TSB-YE	10.0	0.254	Fresh TSB-YE	10.0	0.254

ND=Not detected

The growth of bacteria in "partially" spent broth and neutralised "partially" spent broth exhausted all glucose, thus glucose and lactate readings were not taken as frequently as the first nutrient-supplemented treatments. Growth was still observed in

the glucose depleted suggesting that a substrate other than glucose was used for growth (Fig. 4.20 and 4.21) or that residual, undetectable levels of glucose remained. The MPD was ~10^8 CFU.mL^{-1} in 'partially spent' broths from an initial inoculum of 10^5CFU.mL^{-1} (Fig. 4.17). A similar pattern was observed with *L. monocytogenes* grown in *Le. mesenteroides* spent broth adjusted to pH7 (Figure 4.17).

Rapid uptake of glucose appeared to occur near the start of stationary phase (~ 20 h) for nutrient-supplemented 'partially spent' broths (Figure 4.18 and 4.19). Once the bacterial yield had reached ~10^9 CFU.mL^{-1} rapid depletion of glucose was observed as would be expected from the stoichiometry of exponential growth (see Table 4.7). To better depict the relationship between glucose utilisation rate and microbial growth, log(glucose) and log(lactate) concentrations were plotted against log(viable count).

Table 4.7 The theoretic utilisation of glucose during bacterial growth in TSB-Ye

Bacterial density (CFU.mL^{-1})	**Bacterial level in whole numbers** (CFU.mL^{-1})	**% glucose required for each GT**	**Amount of glucose used from 10 mM**	**Growth-Figures 4.18, 4.19, 4.23 and 4.24**
10^4	10,000	0.001	0.0001 mM	Not observed
10^5	100,000	0.01	0.001 mM	Not observed
10^6	1,000,000	0.1	0.01 mM	Not observed
10^7	10,000,000	1	0.1 mM	Not observed
10^8	100,000,000	10	1 mM	Observed
10^9	1,000,000,000	100	10 mM	Observed

The addition of dehydrated culture media provided additional glucose and allowed the MPD *L. monocytogenes* grown in 'partially spent' broths to reach similar MPDs as growth in "Fresh" TSB-Ye (i.e. ~10^9CFU.mL^{-1}; Figure 4.18 and Figure 4.19). Glucose uptake was observed as lactate increased and pH decreased. As mentioned above the spent broth with no added glucose reached an MPD of 10^8CFU.mL^{-1}, significantly less than the MPD of "Fresh" TSB-Ye.

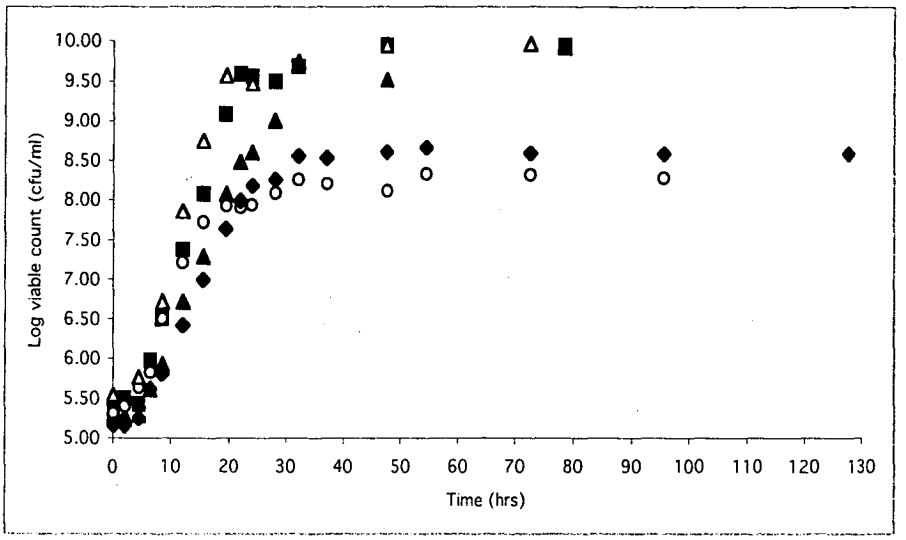


Figure 4.17a Growth of *L. monocytogenes* in *Le. mesenteroides* 'partially spent' broth, where (■) is pH7/TSB-Ye, (▲) is TSB-Ye/altered to spent pH (pH 5.95), (○) is 'partially spent' broth adjusted to pH7, (♦) spent (pH 5.94) and (Δ) is the control (*L. monocytogenes* grown in fresh TSB-Ye broth).

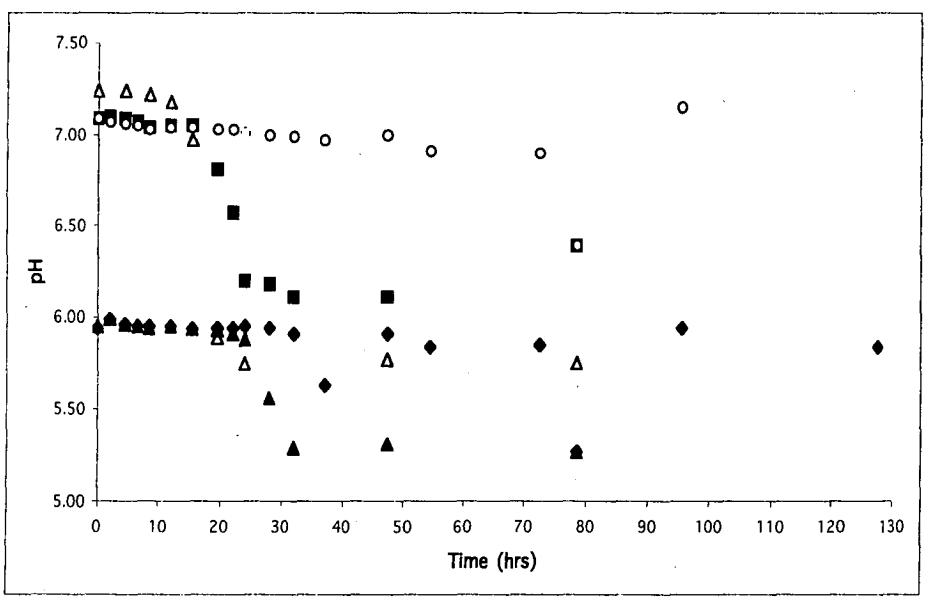


Figure 4.17b pH changes during growth of *L. monocytogenes* in *Le. mesenteroides* 'partially spent' broth where (■) is pH7/TSB-Ye, (▲) is TSB-Ye/pH altered to spent pH(pH 6.39), (○) is broth adjusted to pH7, (♦) is spent (pH 6.39) and (Δ) is the control (*L. monocytogenes* grown in fresh TSB-Ye broth).

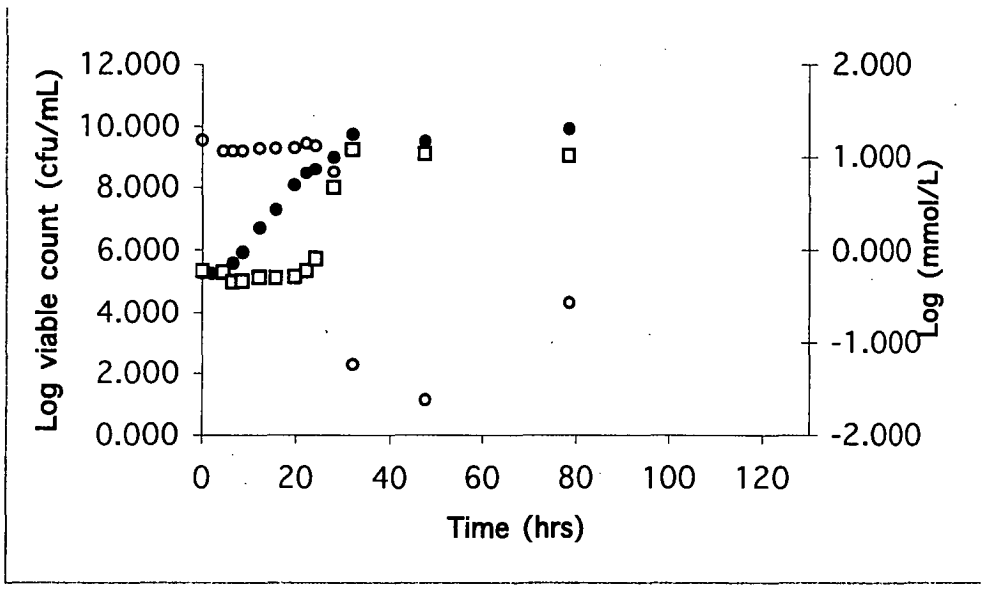


Figure 4.18 Glucose and lactate levels in *L. monocytogenes* cultures grown in *Le mesenteroides* 'partially spent' spent broth with the addition of TSB-Ye and pH adjusted to pH 7 at 25 °C where (●) is growth of *L. monocytogenes* (log viable count ($CFU.mL^{-1}$), (○) glucose) level (log (mM)) and (□) lactate level (log (mM)).

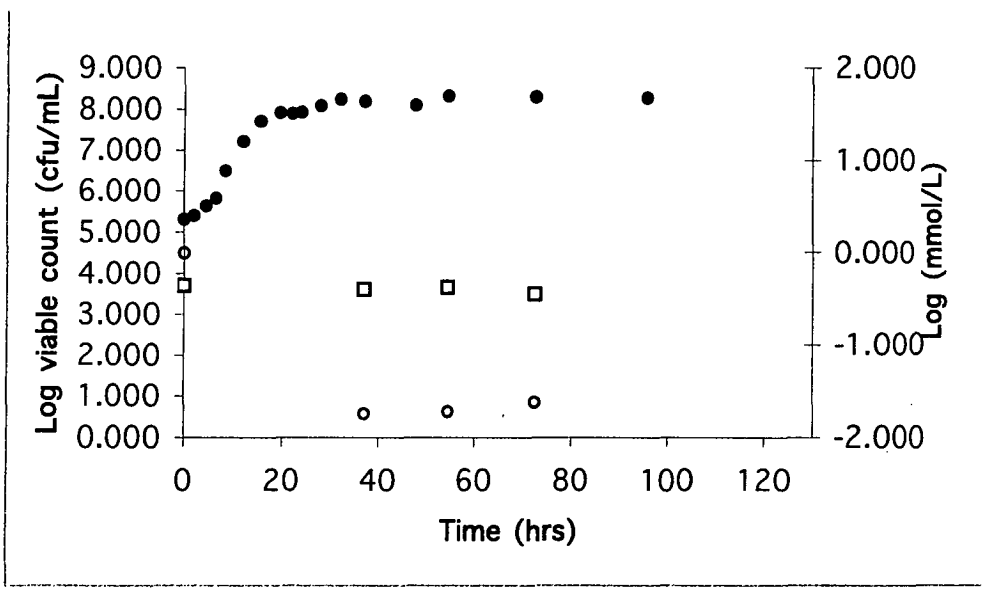


Figure 4.19 Glucose and lactate levels in *L. monocytogenes* cultures grown in *Le. mesenteroides* 'partially spent' broth with the addition of TSB-Ye and pH adjusted to pH 5.95 at 25 °C where (●) is growth of *L. monocytogenes* (log ($CFU.mL^{-1}$), (○) glucose level (log (mM)) and (□) lactate level (log (mM)).

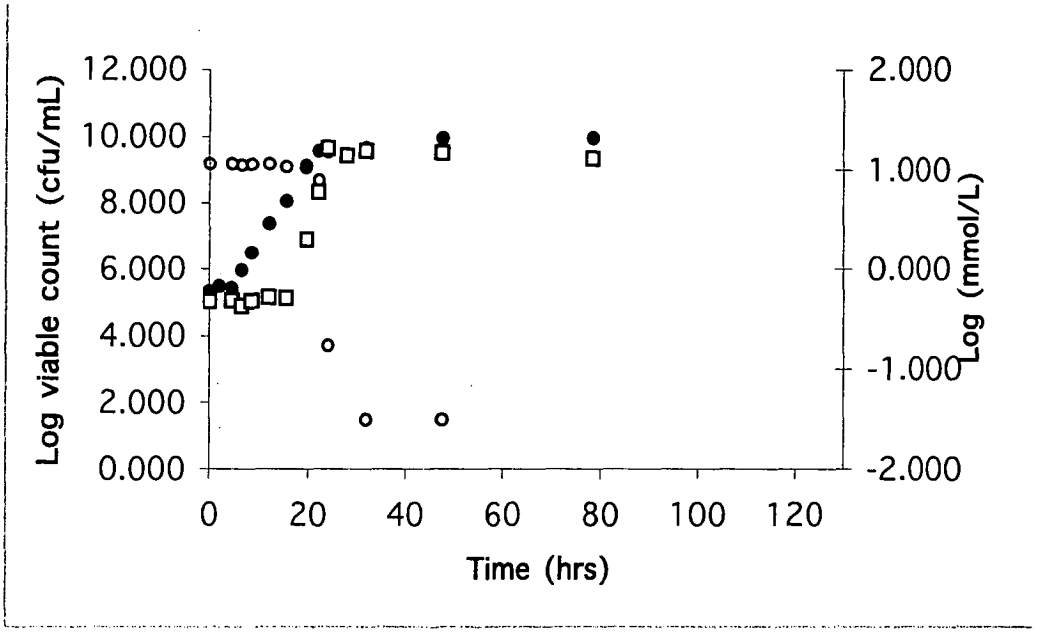


Figure 4.20 Glucose and lactate levels during growth of *L. monocytogenes* in *Le. mesenteroides* 'partially spent' broth with the pH adjusted to pH 7 at 25 °C where (●) is growth of *L. monocytogenes* (log (CFU.mL^{-1}), (○) glucose (log (mM)) and (□) lactate level (log (mM)).

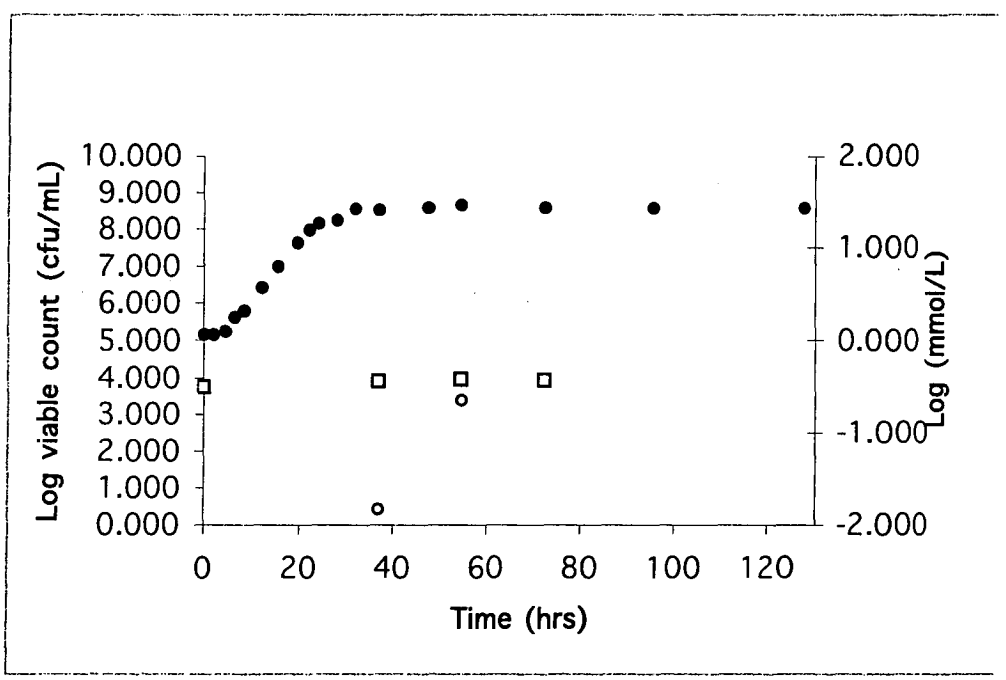


Figure 4.21 Glucose and lactate levels during growth of *L. monocytogenes* in *Le. mesenteroides* 'partially spent' spent broth (pH 5.95) at 25°C where (●) is growth of *L. monocytogenes* (log (CFU.mL^{-1}), (○) glucose (log (mM)) and (□) lactate (log (mM)).

Growth of *L. monocytogenes* in 'partially spent' broths generated from *Lb. sakei.*

Similar results to those reported above were observed for *L. monocytogenes* grown in *Lb. sakei* 'partially spent' broth. In summary the MPD for nutrient supplemented 'partially spent' broths was between 9 to 10 log ($CFU.mL^{-1}$) and the MPD for unamended 'partially spent' and pH7 'partially spent' broth was between 8 and 9 log $CFU.mL^{-1}$ (Table 4.5). Growth curves and changes in media composition are shown in Figures 4.22 to 4.26.

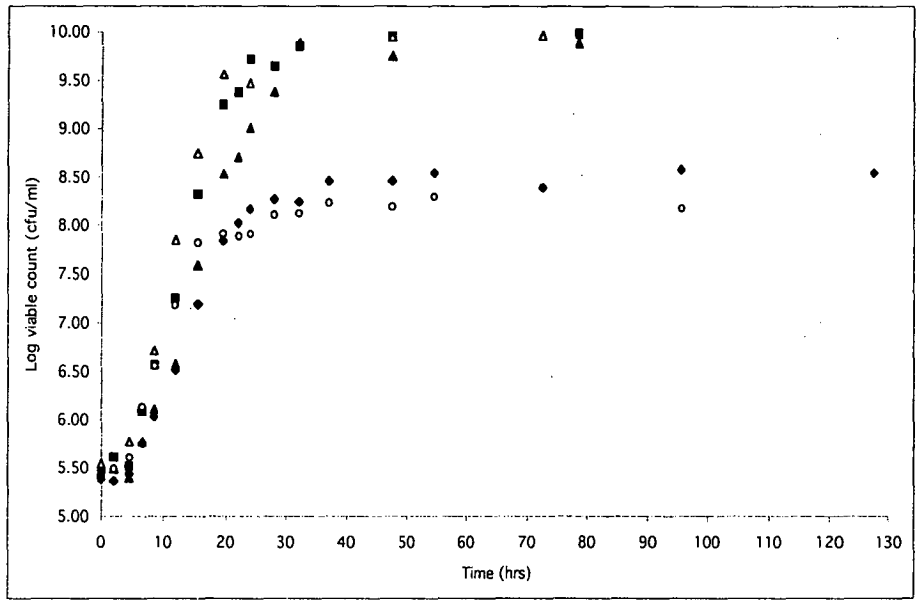


Figure 4.22a Growth of *L. monocytogenes* in *Lb. sakei* 'partially spent' broth where (■) is pH7/TSB-Ye, (▲) is TSB-Ye/altered to 'partially spent' pH (pH 6.02), (○) is broth adjusted to pH7, (♦) is 'partially spent' (pH 5.97) and (Δ) is the control (*L. monocytogenes* grown in fresh TSB-Ye broth).

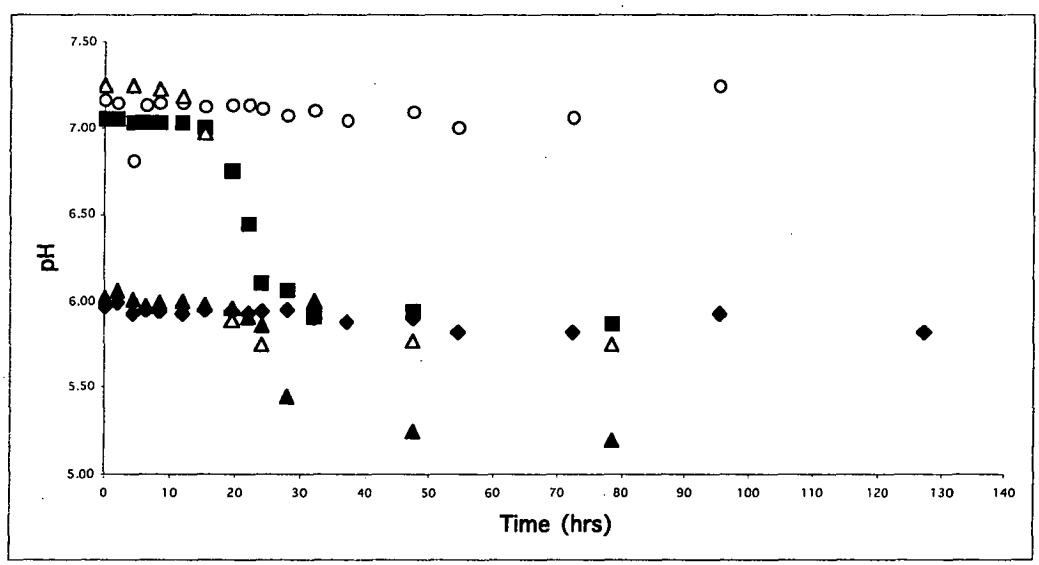


Figure 4.22b pH changes during growth of *L. monocytogenes* in *Lb. sakei* 'partially spent' broth where (■) is pH7/TSB-Ye, (▲) is TSB-Ye/pH altered to 'partially spent' pH(pH 6.39), (○) is 'partially spent' broth adjusted to pH7, (♦) is 'partially spent' (pH 6.39) and (Δ) is the control (*L. monocytogenes* is grown in fresh TSB-Ye broth).

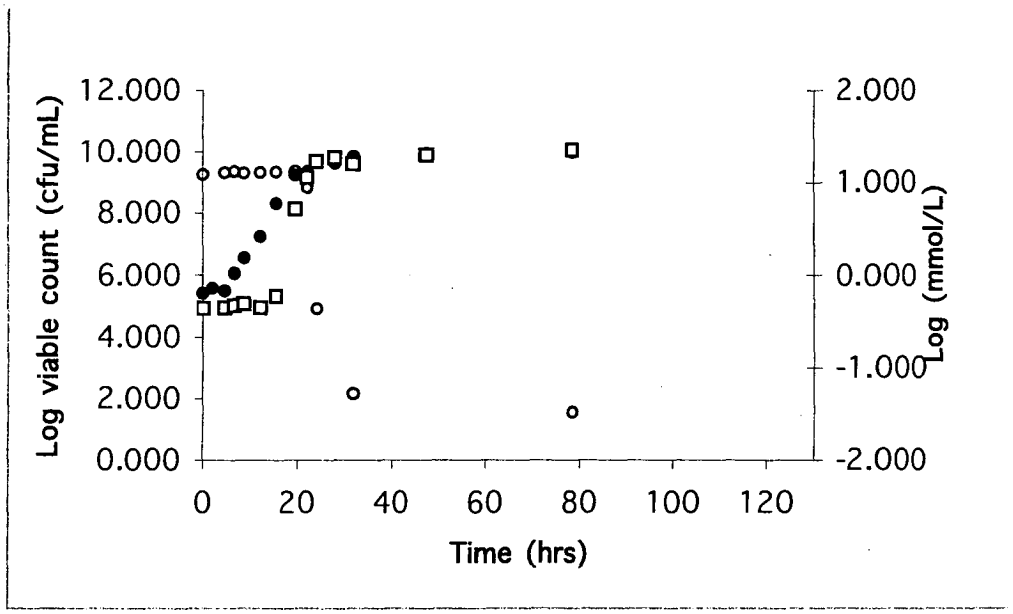


Figure 4.23 Glucose and lactate levels of *L. monocytogenes* batch culture grown in *Lb. sakei* 'partially spent' broth with the addition of TSB-Ye and pH adjusted to pH 7 at 25 °C where (●) is growth of *L. monocytogenes* (log (CFU.mL^{-1}), (○) glucose (log (mM)) and (□) lactate level (log (mM)).

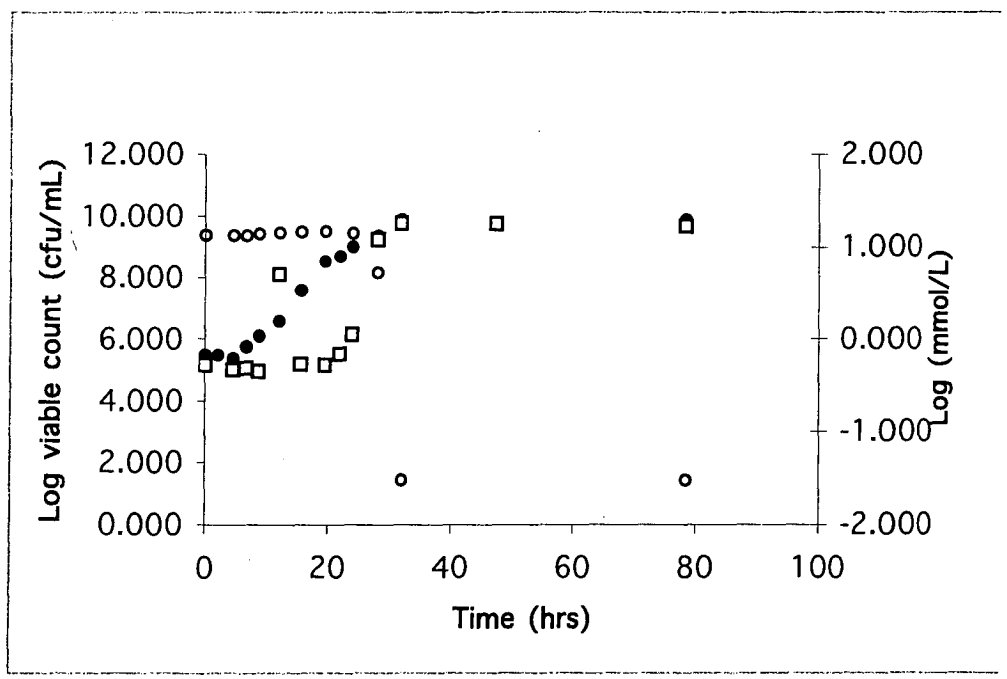


Figure 4.24 Glucose and lactate levels of *L. monocytogenes* grown in *Lb. sakei* 'partially spent' broth with the addition of TSB-Ye and pH adjusted to pH 5.95 at 25 °C where (●) is growth of *L. monocytogenes* (log (CFU.mL^{-1}), (○) glucose) level (log (mM)) and (□) lactate level (log (mM)).

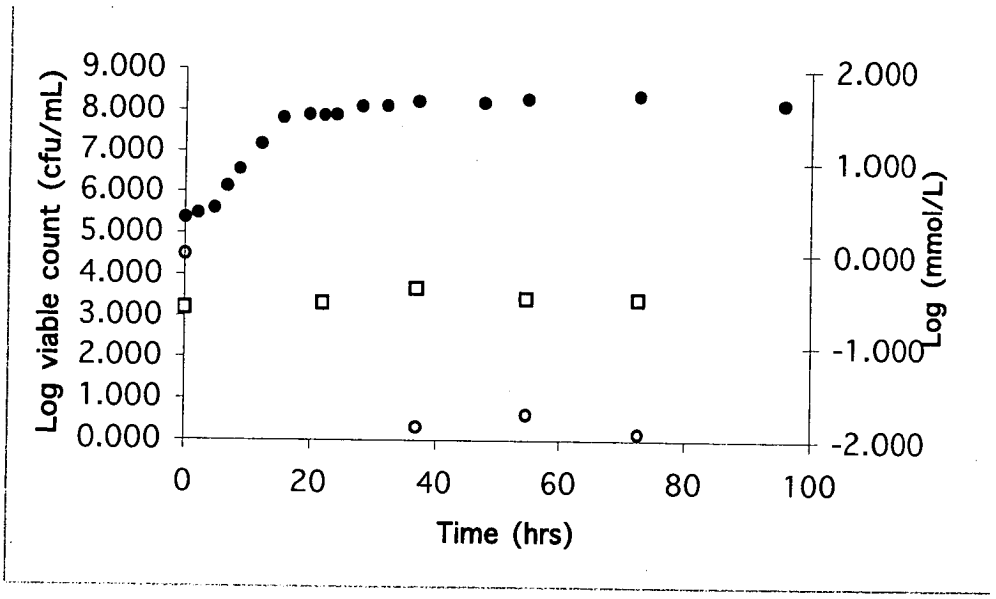


Figure 4.25 Glucose and lactate levels of *L. monocytogenes* grown in *Lb. sakei* 'partially spent' broth with the pH adjusted to pH 7 at 25 °C where (●) is growth of *L. monocytogenes* (log viable count ($CFU.mL^{-1}$), (❍) glucose) level (log (mM)) and (□) lactate level (log (mM)).

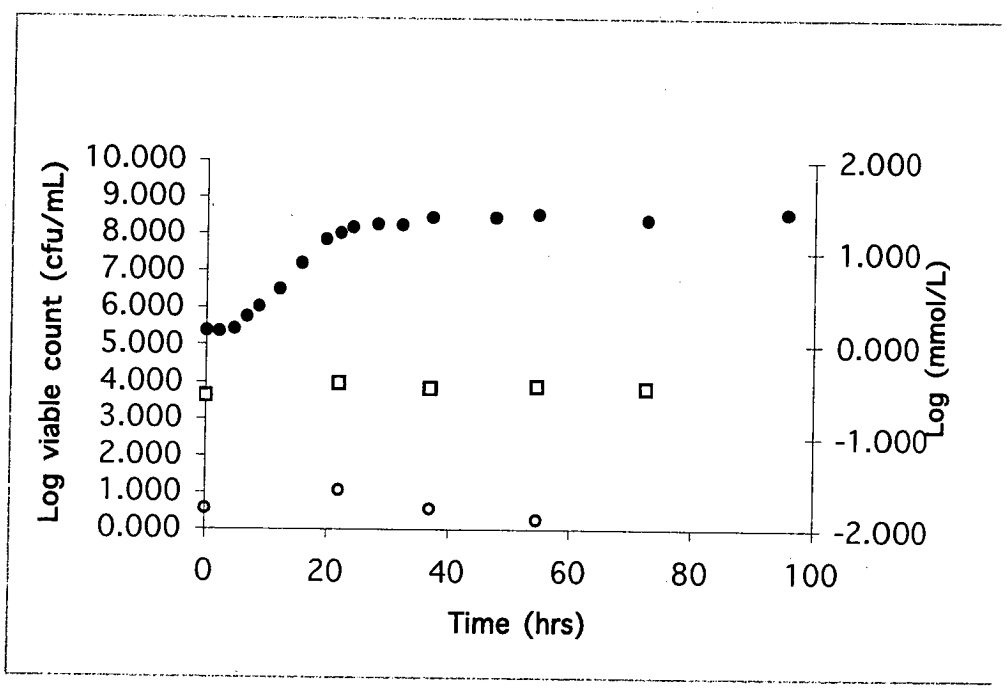


Figure 4.26 Glucose and lactate levels of *L. monocytogenes* grown in *Lb. sakei* 'partially spent' broth (pH 5.95) at 25 °C where (●) is growth of *L. monocytogenes* (log viable count ($CFU.mL^{-1}$), (❍) glucose) level (log mM) and (□) lactate level (log mM).

Growth of *P. fluorescens* in 'partially spent' broths generated from *L. monocytogenes*

One trial was undertaken in which a strain other than *L. monocytogenes*, i.e. *P. fluorescens*, was inoculated into the 'partially spent' broth systems. When *P. fluorescens* was cultured in *L. monocytogenes* 'partially spent' broths, similar trends to that of *L. monocytogenes* cultured in *P. fluorescens* 'spent' broth were observed for most treatments. The nutrient–supplemented broth and 'partially spent' broth adjusted to pH7 reached stationary phase at a similar time (Figure 4.27). Limited data was collected for growth of *P. fluorescens* in the unamended 'partially spent' broth, however, the data available (Fig. 4.27) suggest the MPD was between 7 and 8 log ($CFU.mL^{-1}$). The growth of *P. fluorescens* was slower than *L. monocytogenes* in TSB-Ye thus glucose remained detectable in the broth after 9.5 h of growth (*c.f.* Table 4.2 for *L. monocytogenes* and Table 4.8 for *P. fluorescens*). Table 4.8 shows that there is still > 0.1mM of glucose in TSB-Ye broth after *P. fluorescens* growth for 40 h. Therefore there may have been >0.1mM glucose left after *P. fluorescens* growth in TSB-Ye for 18 h. In addition, no increase in lactate level was observed as glucose was metabolised by *P. fluorescens* after 10 h in TSB-Ye (Figs. 4.6 and 4.7).

Table 4.8 Average (*n*=2 values of %T, pH, glucose and lactate values for TSB-Ye broth during growth of *P. fluorescens*.

Time (h)	%T	pH	Glucose (mM)	Lactate (mM)
0	93.00	7.14	10.40	0.22
1	91.00	7.19	11.00	0.26
3	91.00	7.19	11.15	0.23
4.5	89.50	7.17	10.15	0.26
6.5	68.50	7.09	7.86	0.29
7	55.00	7.07	6.72	0.12
7.5	46.50	7.09	6.38	0.33
8	31.50	7.05	4.49	0.24
8.5	21.00	6.99	2.66	0.20
9.5	9.00	6.91	0.48	0.08
10	7.50	6.90	0.30	0.10
10.5	4.50	6.97	0.14	0.11
11.5	3.25	7.01	0.10	0.07
12.5	2.00	7.04	0.08	0.11
23.5	0.50	7.45	0.12	0.05
41	0.50	8.50	0.20	0.07

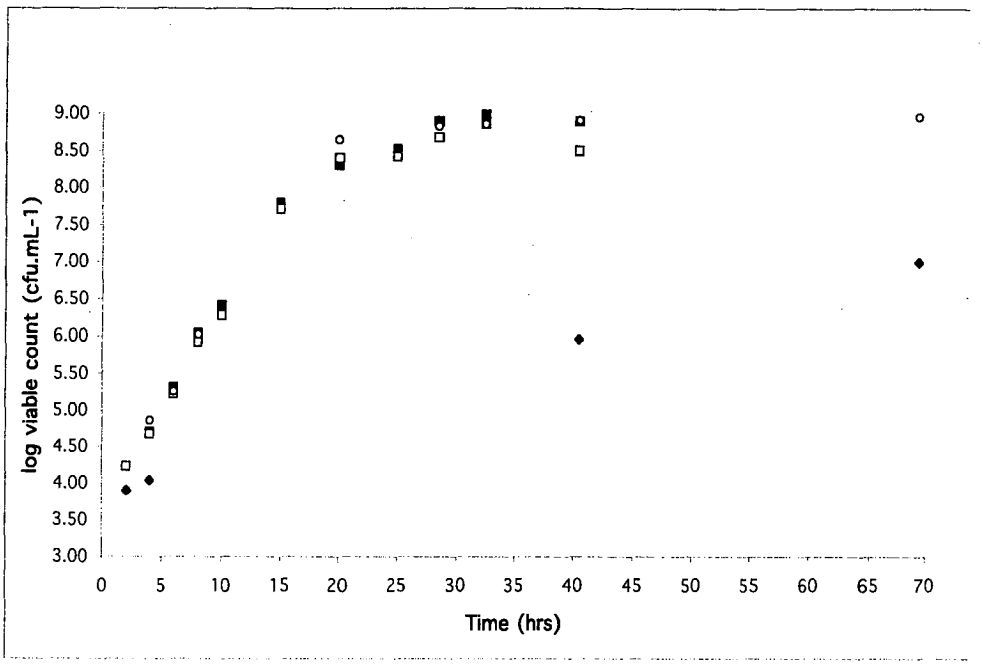


Figure 4.27 Growth of *P. fluorescens* in *L. monocytogenes* 'partially spent' broth where (■) is pH7/TSB-Ye, (□) is TSB-Ye/unaltered pH (pH 6.49), (○) pH7 and (♦) is unamended 'partially spent' (pH 5.39).

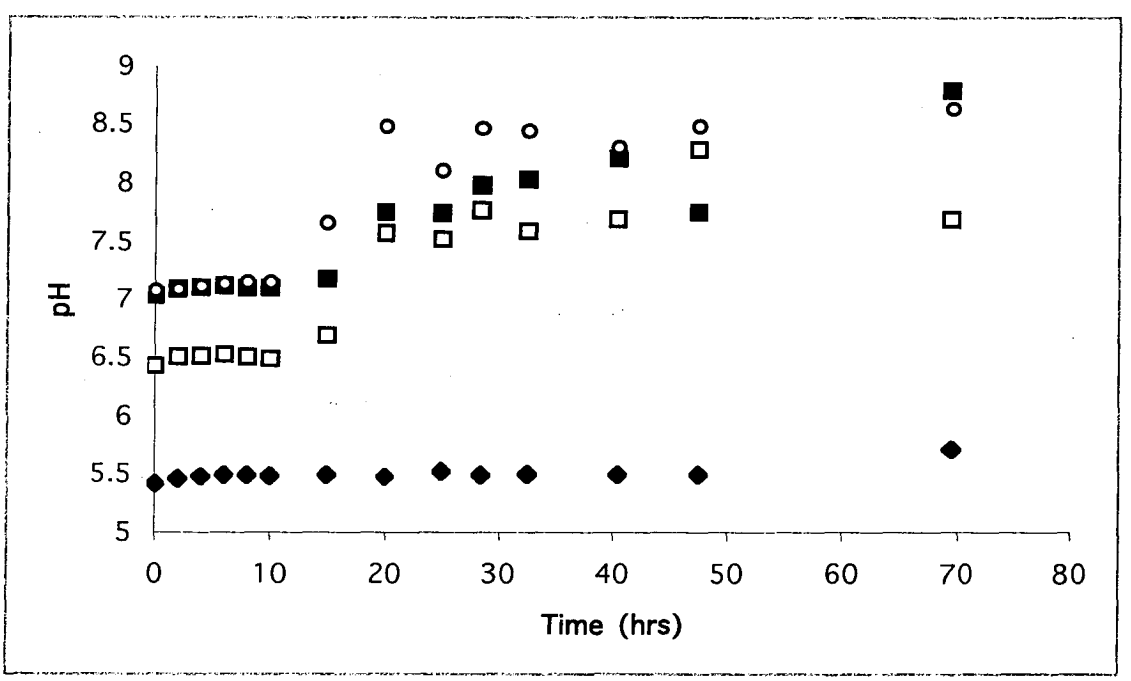


Figure 4.27b pH changes during growth of *P. fluorescens* in *L. monocytogenes* 'partially spent' broth, where (■) is pH7/TSB-Ye, (□) is TSB-Ye/unaltered pH, (○) pH7 and (♦) is 'partially spent'

Growth rate, yield and MPD of various strains in 'spent' and 'partially spent' broth systems

The MPD (Log (CFU.mL^{-1}) was estimated from the average of the highest points on the graphs for each treatment. When *L. monocytogenes* was grown in its own 'spent' or 'partially spent' broth, addition of nutrients allowed the growth of *L. monocytogenes* to reach the same MPD as *L. monocytogenes* grown in fresh TSB-Ye broth, regardless of the pH of the system as seen in the Figures below. A decline in pH resulted when *L. monocytogenes* reached MPD in nutrient-supplemented broth (S_o+N(pH7)) and modified spent broths (S_o+N(pH5.36)), similar to growth in the control (Fo). Little change in pH of 'partially spent' broth was observed when no nutrient was added broth, (S_o (pH7)) even when MPD was reached.

4.3.2.3 *The effect of* L. monocytogenes *growth rate when grown in different spent broths.*

The growth rate of *L. monocytogenes* was plotted against the pH of final treatments for each of the spent broth manipulations listed in Table 4.9 and displayed in Figure 4.28. Also plotted are predictions of *L. monocytogenes* growth rate for each pH value derived from the unpublished model of Ross (T. Ross, *pers. comm.*, 2004). That model includes the following variables, pH, water activity, temperature, nitrate levels and lactate levels and is based on the model of Tienungoon (1998; Eqn. 4.7) supplemented with data obtained in novel experiments and derived from the published literature. Note, however, that the lactic acid levels observed were not used to make growth rate predictions. The points form a continuous curve illustrating the pH effect on growth rate of *L. monocytogenes* in different spent broths.

Most observations lie below the growth rate predicted by the model, and increasingly so at lower pH. This would be consistent with the presence and growth rate-reducing effect of undissociated lactic acid in the culture broths but, as noted above, not included in the model predictions. The model also does not consider the possible effect of other end products of metabolism on growth rate.

Table 4.9a Growth characteristics of *L. monocytogenes* inoculated into various 'spent' and 'fresh' broth systems

Broth System	No.	Figure	Broth system pre-manipulations			Broth systems inoculated with L. monocytogenes				
			Parent culture	MPD	pH	Abbreviations in Text	Symbols	pH-final	GT(h)	MPD
24 h full	1.1	4.9	*L. monocytogenes*	9.28	6.39	S_n+N(pH_)	(□)	6.80	1.60*	9.57
24 h full	1.1	4.9	*L. monocytogenes*	9.28	6.39	S_n(pH7)	(○)	7.27	NC	7.56
24 h full	1.1	4.9	*L. monocytogenes*	9.28	6.39	S_n+N(pH7)	(■)	7.19	1.40*	9.52
24 h full	1.1	4.9	*L. monocytogenes*	9.28	6.39	S_n(pH_)	(◆)	6.39	NC	7.26
24 h full	1.2	4.10	*E. coli*	9.15	6.65	S_n+N(pH_)	(□)	6.89	NC	9.11
24 h full	1.2	4.10	*E. coli*	9.15	6.65	S_n+N(pH7)	(■)	7.21	1.80*	9.86
24 h full	1.2	4.10	*E. coli*	9.15	6.65	S_n(pH_)	(◆)	6.65	2.20	NC
24 h full	1.3	4.11	*P.fluorescens*	8.53	7.22	S_n+N(pH_)	(□)	7.34	1.40*	9.59
24 h full	1.3	4.11	*P.fluorescens*	8.53	7.22	S_n(pH7)	(○)	7.22	1.80*	8.59
24 h full	1.3	4.11	*P.fluorescens*	8.53	7.22	S_n+N(pH7)	(■)	7.22	1.40	9.51
24 h full	1.3	4.11	*P.fluorescens*	8.53	7.22	S_n(pH_)	(◆)	7.22	NC	9.05
18h part	2.2	4.12	*L. monocytogenes*	8.41	5.39	S_n+N(pH7)	(■)	7.15	1.11	8.56
18h part	2.2	4.12	*L. monocytogenes*	8.41	5.39	S_n+N(pH_)	(□)	6.43	1.18	8.63
18h part	2.2	4.12	*L. monocytogenes*	8.41	5.39	S_n(pH_)	(◆)	5.39	NC	NC
18h part	3.1	4.13	*L. monocytogenes*	8.59	5.54	S_n(pH7)	(○)	7.11	NC	7.06
18h part	3.1	4.13	*L. monocytogenes*	8.59	5.54	S_n(pH_)	(◆)	5.54	NC	6.88
18h part	3.1	4.13	*L. monocytogenes*	8.59	5.54	S_n+N(pH-S)	(▲)	5.84	4.40*	7.96
18h part	4.1	4.14	*L. monocytogenes*	9.15	5.36	S_n(pH7)	(○)	7.49	1.40*	8.53
18h part	4.1	4.14	*L. monocytogenes*	9.15	5.36	S_n+N(pH7)	(■)	7.20	1.14	9.91
18h part	4.1	4.14	*L. monocytogenes*	9.15	5.36	S_n(pH_)	(◆)	5.36	2.70	8.01
18h part	4.1	4.14	*L. monocytogenes*	9.15	5.36	S_n+N(pH-S)	(▲)	5.57	1.79	9.73
18h part	4.2	4.15	*L.mono. (low inoculum)*	9.15	5.36	S_n(pH_)	(◆)	5.36	3.90	8.28
18h part	4.2	4.15	*L.mono. (low inoculum)*	9.15	5.36	S_n+N(pH-S)	(▲)	5.57	2.35	9.53
18h part	4.3	4.16	*E.coli*	9.18	6.39	S_n(pH_)	(◆)	6.39	3.40	8.81
18h part	4.3	4.16	*E.coli*	9.18	6.39	S_n(pH7)	(○)	7.40	1.90	NC
18h part	4.3	4.16	*E.coli*	9.18	6.39	S_n+N(pH7)	(■)	7.06	1.20*	9.79
18h part	4.3	4.16	*E.coli*	9.18	6.39	S_n+N(pH-S)	(▲)	6.39	1.50*	9.76
18h part	5.1	4.17	*Le. mesenteroides*	8.60	5.95	S_n(pH7)	(○)	7.00	1.50*	8.31
18h part	5.1	4.17	*Le. mesenteroides*	8.60	5.95	S_n(pH_)	(◆)	5.94	2.00	8.30
18h part	5.1	4.17	*Le. mesenteroides*	8.60	5.95	S_n+N(pH-S)	(▲)	5.95	1.70	9.85
18h part	5.1	4.17	*Le. mesenteroides*	8.60	5.95	S_n+N(pH7)	(■)	7.09	1.30*	9.86
18h part	5.2	4.22	*Lacto. sakei*	8.59	5.96	S_n(pH7)	(○)	7.16	1.50*	8.30
18h part	5.2	4.22	*Lacto. sakei*	8.59	5.96	S_n+N(pH7)	(■)	7.05	1.40*	9.93
18h part	5.2	4.22	*Lacto. sakei*	8.59	5.96	S_n(pH_)	(◆)	5.94	1.90	8.61
18h part	5.2	4.22	*Lacto. sakei*	8.59	5.96	S_n+N(pH-S)	(▲)	6.02	1.80	9.89
Control	4.1	4.14, 4.16	Growth in unamended TSB-Ye			F_n	(Δ)	7.27	1.11	9.88
Control	5.1	4.17, 4.22	Growth in unamended TSB-Ye			F_n	(Δ)	7.24	1.16	9.80

Where * denotes data 'less-than', and shaded values denote 'outliers' compared with predicted growth of *L. monocytogenes* (Fig. 4.28). NC=not calculated. All other symbols and abbreviations are as described in Table 4.4.

Table 4.9b Data from table 4.9 (a) sorted by broth treatment

Broth System	No.	Figure	Broth system pre-manipulations			Broth systems inoculated with L. monocytogenes				
			Parent culture	MPD	pH	Abbreviations in Text	Symbols	pH-final	GT(h)	MPD
18h part	4.3	4.16	*E.coli*	9.18	6.39	So(pH_)	(◆)	6.39	3.40	8.81
18h part	2.2	4.12	*L. monocytogenes*	8.41	5.39	So(pH_)	(◆)	5.39	NC	NC
18h part	3.1	4.13	*L. monocytogenes*	8.59	5.54	So(pH_)	(◆)	5.54	NC	6.88
18h part	4.1	4.14	*L. monocytogenes*	9.15	5.36	So(pH_)	(◆)	5.36	2.70	8.01
18h part	4.2	4.15	*L.mono. (low inoculum)*	9.15	5.36	So(pH_)	(◈)	5.36	3.90	8.28
18h part	5.2	4.22	*Lacto. sakei*	8.59	5.96	So(pH_)	(◆)	5.94	1.90	8.61
18h part	5.1	4.17	*Le. mesenteroides*	8.60	5.95	So(pH_)	(◆)	5.94	2.00	8.30
24 h full	1.2	4.10	*E.coli*	9.15	6.65	So(pH_)	(◆)	6.65	2.20	NC
24 h full	1.1	4.9	*L. monocytogenes*	9.28	6.39	So(pH_)	(◆)	6.39	NC	7.26
24 h full	1.3	4.11	*P.fluorescens*	8.53	7.22	So(pH_)	(◆)	7.22	NC	9.05
18h part	4.3	4.16	*E.coli*	9.18	6.39	So(pH7)	(○)	7.40	1.90	NC
18h part	3.1	4.13	*L. monocytogenes*	8.59	5.54	So(pH7)	(○)	7.11	NC	7.06
18h part	4.1	4.14	*L. monocytogenes*	9.15	5.36	So(pH7)	(○)	7.49	1.40*	8.53
18h part	5.2	4.22	*Lacto. sakei*	8.59	5.96	So(pH7)	(○)	7.16	1.50*	8.30
18h part	5.1	4.17	*Le. mesenteroides*	8.60	5.95	So(pH7)	(○)	7.00	1.50*	8.31
24 h full	1.1	4.9	*L. monocytogenes*	9.28	6.39	So(pH7)	(○)	7.27	NC	7.56
24 h full	1.3	4.11	*P.fluorescens*	8.53	7.22	So(pH7)	(○)	7.22	1.80*	8.59
18h part	4.3	4.16	*E.coli*	9.18	6.39	So+N(pH7)	(■)	7.06	1.20*	9.79
18h part	2.2	4.12	*L. monocytogenes*	8.41	5.39	So+N(pH7)	(■)	7.15	1.11	8.56
18h part	4.1	4.14	*L. monocytogenes*	9.15	5.36	So+N(pH7)	(■)	7.20	1.14	9.91
18h part	5.2	4.22	*Lacto. sakei*	8.59	5.96	So+N(pH7)	(■)	7.05	1.40*	9.93
18h part	5.1	4.17	*Le. mesenteroides*	8.60	5.95	So+N(pH7)	(■)	7.09	1.30*	9.86
24 h full	1.2	4.10	*E.coli*	9.15	6.65	So+N(pH7)	(■)	7.21	1.80*	9.86
24 h full	1.1	4.9	*L. monocytogenes*	9.28	6.39	So+N(pH7)	(■)	7.19	1.40*	9.52
24 h full	1.3	4.11	*P.fluorescens*	8.53	7.22	So+N(pH7)	(■)	7.22	1.40	9.51
18h part	2.2	4.12	*L. monocytogenes*	8.41	5.39	So+N(pH_)	(□)	6.43	1.18	8.63
24 h full	1.2	4.10	*E.coli*	9.15	6.65	So+N(pH_)	(□)	6.89	NC	9.11
24 h full	1.1	4.9	*L. monocytogenes*	9.28	6.39	So+N(pH_)	(□)	6.80	1.60*	9.57
24 h full	1.3	4.11	*P.fluorescens*	8.53	7.22	So+N(pH_)	(□)	7.34	1.40*	9.59
18h part	4.3	4.16	*E.coli*	9.18	6.39	So+N(pH-S)	(▲)	6.39	1.50*	9.76
18h part	3.1	4.13	*L. monocytogenes*	8.59	5.54	So+N(pH-S)	(▲)	5.84	4.40*	7.96
18h part	4.1	4.14	*L. monocytogenes*	9.15	5.36	So+N(pH-S)	(▲)	5.57	1.79	9.73
18h part	4.2	4.15	*L.mono. (low inoculum)*	9.15	5.36	So+N(pH-S)	(◭)	5.57	2.35	9.53
18h part	5.2	4.22	*Lacto. sakei*	8.59	5.96	So+N(pH-S)	(▲)	6.02	1.80	9.89
18h part	5.1	4.17	*Le. mesenteroides*	8.60	5.95	So+N(pH-S)	(▲)	5.95	1.70	9.85
Control	4.1	4.14, 4.16	Growth in unamended TSB-Ye			Fo	(Δ)	7.27	1.11	9.88
Control	5.1	4.17, 4.22	Growth in unamended TSB-Ye			Fo	(Δ)	7.24	1.16	9.80

Where * denotes data 'less-than', and shaded values denote 'outliers' compared with predicted growth of *L. monocytogenes* (Fig. 4.28). NC=not calculated. All other symbols and abbreviations are as described in Table 4.

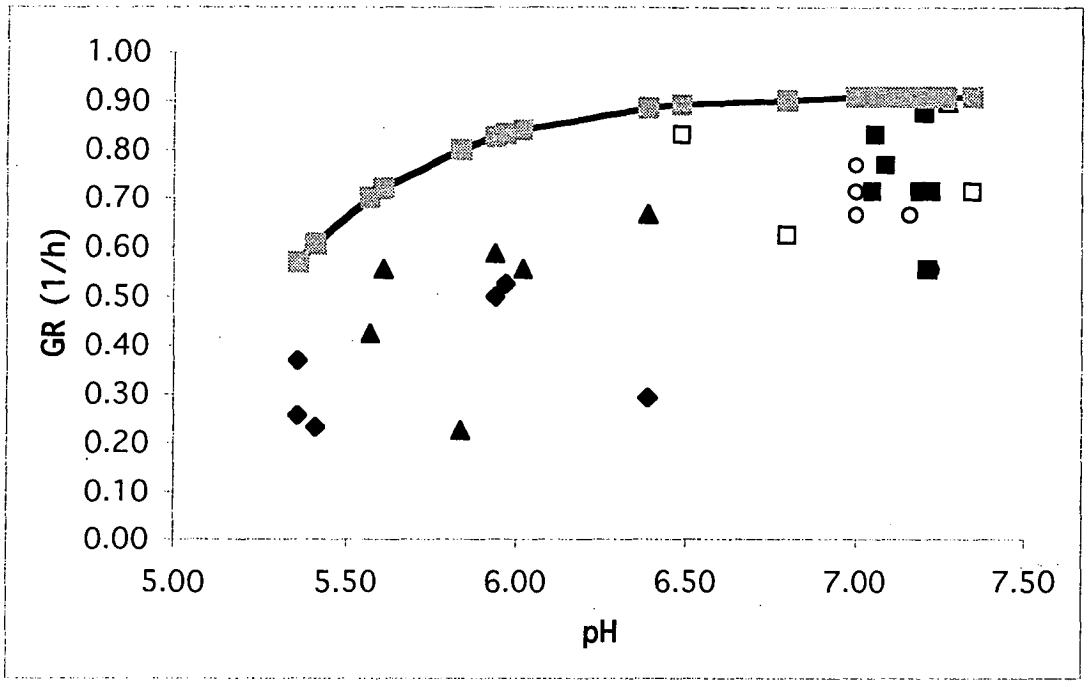


Figure 4.28 Observed (solid symbols) and predicted curve growth rate (~~~~) of *L. monocytogenes* cultured in 'spent' broth systems at various initial pH values where (■) is pH7/TSB-Ye, (□) is TSB-Ye added with no adjustment of the pH (▲) is TSB-Ye/pH altered to spent pH(pH 6.39), (○) is broth adjusted to pH7, (♦) spent (pH 6.39) and (Δ △) is the control where *L. monocytogenes* is grown in fresh TSB-Ye broth.

4.4 Discussion

4.4.1 The effect of nutrient depletion and pH on the suppression of MPD of *L. monocytogenes* grown in different spent broths.

In this study the bacteria of particular interest to producers of cooked, refrigerated, ready to eat meats are the two LAB, *Le. mesenteroides* and *Lb. sakei,* and *L. monocytogenes. E. coli* and *P. fluorescens* were also included to complement and extend the studies of Davidson (*unpublished*) and Mellefont (*unpublished*) also undertaken in this laboratory. Davidson (*unpublished*) inoculated *L. monocytogenes* into its own, *P. fluorescens* and *E. coli* spent broth produced from 20 h cultures but viable counts were determined only after 24 h incubation at 25°C. In this study, Davidson's work was repeated, but with the growth curves monitored intensively. Preliminary experiments using *L. monocytogenes*, *Escherichia coli* M23 and *Pseudomonas fluorescens* spent broth (24 h and 18 h, 25 °C) were all used in the current study to replicate Davidson's results. *Lb. sakei*, *Le. mesenteroides*, *E. coli* and *P. fluorescens* did not produce bacteriocins against *L. monocytogenes* (Scott A) (*see* Chapter 3). All these bacteria were included to assess whether the "Jameson Effect" is caused by specific antimicrobial factors or by other, non-species specific mechanisms. Therefore non-specific factors causing MPD suppression are suggested, and the mechanisms may be explored by growing *L. monocytogenes* in different cell-free spent broths. The possible role of pH and nutrient depletion as mediators of the Jameson Effect were examined by exploring growth of test bacteria in unamended spent broth, nutrient-supplemented spent broth with pH altered to neutrality, nutrient-supplemented spent broth with pH adjusted back to that of unamended spent broth and, unamended spent broth with pH altered to neutral. *L. monocytogenes* growth rate and MPD in TSB-Ye was compared with growth in spent broths to test the hypothesis that the Jameson Effect on growth of *L. monocytogenes* is due to nutrient depletion and /or pH reduction due to the growth of competing organisms.

4.4.2 Effect of Nutrient Depletion

Experiments described above explore factors causing MPD suppression when bacteriocins were not a consideration. Nutrient depletion was suggested to be a non-

specific factor causing *L. monocytogenes* MPD suppression in *E. coli*, *L. monocytogenes*, *Le. mesenteroides* and *Lb. sakei* spent broths. There is limited literature available on the outcome of *L. monocytogenes* grown in different bacterial spent broths however several competition trials using *L. monocytogenes* in co-culture have been reported (Breidt and Fleming, 1998, Buchanan and Bagi, 1999, Szigeti, 2001 and Farkas *et al.*, 2002). For example, bacteriocin negative strains of *C. piscicola* (Nilsson *et al.*, 1999) and *Lactobacillus casei* and *Lactobacillus paracasei* (Amezquita and Brashears, 2002) were able to suppress *L. monocytogenes* in co-culture on cold smoked salmon and ready-to-eat meat respectively.

The effects of low pH in spent broth caused by bacterial metabolism after MPD is reached, was investigated in this study independently from nutrient depletion. These two factors were identified individually from experiment 3.1 onwards (Table 4.5). Treatments involving adding nutrients and adjusting pH to spent pH were undertaken to identify the effect of nutrient depletion alone on the MPD of *L. monocytogenes*. This treatment was compared to the treatment where the pH was adjusted to neutral and media added. *L. monocytogenes* growth in this 'partially' spent broth took 20 h to reach MPD (Fig. 4.9a). The 'partially' spent pH treatment reached maximum population by about 50 h (Figure 4.9a) indicating that low pH may cause a longer lag phase however the MPD was similar to *L. monocytogenes* growing in fresh TSB-Ye broth (Table 4.5). Neutral pH is advantageous to most bacteria, including *L. monocytogenes*, imposing fewer hurdles to growth rate so that the MPD is reached earlier than *L. monocytogenes* grown in a low pH environment. MPD similar to that observed in "Fresh" TSB-Ye broth was reached for all broths with added nutrient irrespective of pH indicating that pH did not effect the level of MPD reached (Table 4.5). This finding was supported by Leroy and de Vuyst (2001) who concluded that a nutrient depletion model was more useful than the logistic growth model when taking into account different environmental conditions.

When nutrient was added and pH was lowered to spent pH, the MPD still reached ~10^9 CFU.mL^{-1} at stationary phase, which was the MPD reached when *L. monocytogenes* was grown in "Fresh" TSB-Ye broth. If nutrient were not added to spent broth, MPD was between 10^7 to 10^8 CFU.mL^{-1}. The end products of

metabolism in spent broths did not prevent *L. monocytogenes* attaining the MPD of "Fresh" TSB-Ye broth, indicating that nutrient depletion is the main factor limiting MPD in the systems studied and as a mechanism of the Jameson Effect. Most MPD differences were between 1 and 1.5 logs (Table 4.5) in spent broths compared to Fresh broths or spent broths supplemented with additional nutrients. A higher MPD difference than 1.5 logs was reached for *Lb. sakei*, *Le. mesenteroides* and *E. coli* spent broth adjusted to pH neutral. Addition of nutrient to spent broth had a significant effect on MPD reached whereas pH had a minor influence on MPD.

4.4.3 Effect of spent pH

'Spent' pH (i.e. low pH) caused *L. monocytogenes* to reach MPD at a slower rate however a similar MPD was reached by *L. monocytogenes* grown in 'spent' or 'partially' spent broth with added nutrient and pH adjusted to spent pH and *L. monocytogenes* grown in fresh TSB-Ye. The delay in reaching MPD compared to 'spent' broth with added nutrient and pH adjusted to 'spent' pH, may have been due the low pH causing *L. monocytogenes* to have a lag phase (Mellefont, 2000) as well as a slower growth rate. This observation is contradicted by Breidt and Fleming (1998) who suggested the main cause of suppression of *L. monocytogenes* MPD was a low pH of 4 created by *L. lactis* in co-culture with *L. monocytogenes* (Section 1.12.4.2), although at near growth limiting conditions (such as pH 4 for *L. monocytogenes*), reduction of cell yield has been observed by others (e.g. Krist *et al.*, 1998). The pH of spent broths in this study are not near to *L. monocytogenes* growth limits (*see* Section 1.10). Other authors suggest the production of organic acids from substrate utilisation as the cause of the suppression of MPD of *L. monocytogenes* by *Lactobacillus casei* and *Lactobacillus paracasei* in Amezquita and Brashears (2002). In Amezquita and Brashears (2002) study, the cell-free supernatants were adjusted to pH 6.50 to eliminate the effect of week organic acids. Organic acid production (lactate) was measured in this study.

4.4.4 Inoculum level

The effect of *L. monocytogenes* inoculum level on MPD was measured by inoculating two different levels of *L. monocytogenes* into *L. monocytogenes* spent broth (i.e. ~10^5

and ~10^3 CFU.mL^{-1}). "High inoculum" treatments took 20 h and "low inoculum" treatments took 55 h to reach stationary phase with the same MPD observed in either case. This observation may suggest that inoculum size does not influence *L. monocytogenes* MPD in monoculture. This was also seen in the spent broth where the lower inoculum treatment showed bacteria to eventually reach the same level as the higher inoculum bacteria treatment (Table 4.5). However these observations are only speculative as more data is required for a statistical conclusion. There was little difference between the pH of spent broths for both inoculum levels (Figure 4.15b).

4.4.5 Interactions between *L. monocytogenes* and *P. fluorescens.*

Conversely, when *L. monocytogenes* was grown in *P. fluorescens* partially spent broth, all treatments grew equally well including the unamended spent broth. All treatments had a close to neutral initial pH (Fig. 4.11b). Addition of nutrient to *P. fluorescens* 'partially spent' broth made no observable difference to the growth of *L. monocytogenes* in different treatments at 25 °C. A similar trend was also observed for *P. fluorescens* grown in *L. monocytogenes* spent broth however the spent broth in that experiment, had a lower MPD (i.e. 2 log lower than 'partially spent' broth, pH neutral treatments and treatments with added TSB-Ye) (Fig. 4.27). The pH of the second spent broth was 5.39 which was low, and *P. fluorescens* has a relatively high minimum pH for growth (Kamperman, 1994). Different nutrients may be preferred by *L. monocytogenes* and *P. fluorescens* as unamended 'partially spent' broth did not prevent the growth of *L. monocytogenes*. Verheul *et al.* (1995) stated the importance of other micro-organisms on meat surfaces to provide peptides from protein for *L. monocytogenes* growth. Proteolytic and non-proteolytic *P. fluorescens* were studied in co-culture by Worm *et al.* (2000). Worm *et al.* (2000) studied growth of proteolytic and non-proteolytic strains of *P. fluorescens* in a nitrogen deficient medium. The protein hydrolysates produced by the proteolytic strains were available to be used by both strains. This may explain the growth advantage of *L. monocytogenes* when grown in competition with *P. fluorescens* in the current study. *P. fluorescens* may use different substrates and/or provide peptides that assist the growth of *L. monocytogenes*. The proteolytic ability of *P. fluorescens* (44) used in this study was not examined. Nutrients remained in the *P. fluorescens* spent broth that *L. monocytogenes* was able to use. Buchanan and Bagi (1999) noted a slight

stimulation of growth of *L. monocytogenes* in co-culture with *P. fluorescens* when grown in BHI at 12°C, 25 $g.L^{-1}$ NaCl and pH 5. Buchanan and Bagi (1999) also note that *L. monocytogenes* was inhibited at low temperature (4°C). Higher temperatures favour *L. monocytogenes* growth. This was also seen by del Campo *et al.* (2001) where *L. monocytogenes* was grown in co-culture with *P. fluorescens* and *Enterobacteriaceae* at 10°C. In the current study there was no suppression of *L. monocytogenes* MPD in co-culture with *P. fluorescens*.

The release of nutrients by *P. fluorescens* can be related to the growth of *L. monocytogenes* on the surface of meat products. Verheul *et al* (1995) suggested that there were not enough amino acids on meat for growth of a mono-culture of *L. monocytogenes*. The low free amino acid level of meat has lead researchers to question why *L. monocytogenes* still grows well on meats. The presence of other microorganisms may provide free amino acids for *L. monocytogenes* to utilise and grow. The proportion of essential free amino acids on fresh meat are 0.1%-0.3% (Beumer *et al.*, 1994). It is suggested by Beumer *et al.* (1994) that amino acid levels of 0.1%-0.3% are enough for growth, apparently contradicting the results of Verheul *et al* (1995). However if meat does not contain all essential amino acids needed for *L. monocytogenes* growth as free amino acids, then Verheul *et al* (1995) could be correct. This may help explain the observations found in this study regarding *P. fluorescens* as unsuitable as a competitor against *L. monocytogenes* on VP/MAP meat. Moreover, as a strict aerobe, *P. fluorescens* growth would expected to be inhibited in such products.

4.4.6 Growth rate

The relationship between pH and growth rate is depicted in Figure 4.28. The growth rate of *L. monocytogenes* was affected by the pH of the broth regardless of the addition of nutrient. On the other hand, MPD was affected by nutrient depletion regardless of the pH of the broth (Table 4.5). Several points lie at a greater distance from the predicted curve. These points represent *L. monocytogenes* growth in spent broths of *E. coli* (pH 6.39), *P. fluorescens* (pH 7.22) and *L. monocytogenes* (pH 5.84) which had been adjusted to pH 7 with no added nutrients. *L. monocytogenes* spent broth with pH adjusted to spent pH also fell some distance from the predicted curve.

The end products of substrate metabolism by LAB, *E. coli* and *P. fluorescens* may differ from glucose metabolism by *L. monocytogenes* (i.e. lactate production from glucose). End products of *E. coli* and *P. fluorescens* metabolism may slow the growth rate of *L. monocytogenes* (Jason, 1983).

4.4.7 Glucose level

It was established (Section 4.4.2) that nutrient depletion was the main cause of MPD suppression when *L. monocytogenes* is in co-culture with *E. coli*, *Le. mesenteroides* and *Lb. sakei*. The main substrate utilised by *L. monocytogenes* is glucose (Section 1.8.2.1) therefore glucose utilisation was measured to observe the effects of glucose depletion. Glucose is present in TSB-Ye (10 $g.L^{-1}$) thus glucose and lactate levels of *L. monocytogenes* growing in LAB spent broths were monitored throughout the experiment. Lactate was measured to establish an insight into what biochemical pathways *L. monocytogenes* and the LAB are using when metabolising glucose. This study is concerned with VP/MAP ready-to-eat meat thus knowledge of the glucose content of this product may be instrumental when determining bacterial interactions, or the potential for microbial growth.

4.4.7.1 MRS and TSB-Ye

L. monocytogenes, Le. mesenteroides and *Lb. sakei* were grown in MRS and TSB-Ye to determine the optimal growth medium for both LAB species and *L. monocytogenes*. *L. monocytogenes* and the two LAB grew equally well in TSB-Ye and LAB grew equally as well in TSB-Ye as in MRS, but *L. monocytogenes* did not grow in MRS. Therefore LAB was grown in TSB-Ye to produce LAB 'partially spent' broths.

Glucose metabolism in both broths resulted in lactate production by *L. monocytogenes* as expected however no lactate was produced by the LAB (Table 4.2). This may be due to the aerobic environment of the flasks. The lactate pathway is not favoured by LAB when the environment is aerobic (Adams and Moss, 2000). The LAB growing in an aerobic environments, may favour the pathway for acetate production as oxygen

is available thus less stored energy (ADP) is required to make ATP (Adams and Moss, 2000).

In this study the pH was lower in MRS broth after the growth of LAB as more glucose was available. MPD was reached after 9.5 h at 30°C however glucose was not fully depleted. This may be due to a low pH unfavourable to the LAB as a result of a higher production of organic acids. The higher the consumption of glucose, the more organic acid produced and the lower the pH. The optimum pH and temperature for *Le. mesenteroides* and *Lb. sakei* were reported by Mataragas *et al* (2003 and Petaja *et al.* (2003) to be 6-6.5 and 30°C respectively. In this study MRS broth pH was 5.0 and 4.7 after growth with *Le. mesenteroides* and *Lb. sakei* respectively (Table 4.1) and, thus, may have prevented the LAB from reaching a higher yield.

Leroy and de Vuyst (2001) studied growth of *Lb. sakei* CTC 494 in MRS broth at 20, 25 and 30 °C and pH 5.5 and 6.5 to examine why bacteria are self-limiting in MRS broth when the broth has been designed specifically for LAB growth. The factors investigated included lactic acid formation and nutrient depletion. The maximum concentration of undissociated lactic acid that permits growth of *Lb. sakei* is ±2.7 mM. MRS contains 20g/L of glucose which equates to 0.45g.L^{-1} of undissociated lactic acid at pH 5.5. This level is lower (0.05g/L) when the pH is 6.5. Thus, undissociated lactic acid alone did not explain the inhibition at pH6.5 when 99.8% of lactic acid is expected to be dissociated.

4.4.7.2 Glucose level in Nutrient added broths

Glucose was utilised in LAB spent broth within 20 to 40 h after *L. monocytogenes'* MPD had been reached (Figures 4.18, 4.20, 4.23 and 4.24). Lactate levels increased as glucose levels decreased as expected with *L. monocytogenes* metabolism of glucose (Trivett and Meyer, 1971; Premaratne *et al.*, 1991).

4.4.7.3 Glucose in LAB spent broth

Growth was observed in spent broth even though limited glucose was available in spent broth. The substrates utilised by *L. monocytogenes* during nutrient depletion, have been studied in detail by Jones and Kompala (1999) and Herbert and Foster (2001).

In cybernetic theory (Patnaik, 2000), bacteria are expected to use the biochemical pathway that leads to fastest specific growth rate. Jones and Kompala (1999) studied *Saccharomycoses cerevisiae* grown in continuous culture and monitored glucose, ethanol, specific growth rate and cell mass. Diauxic behaviour was observed during the growth of the microorganism. Glucose was consumed and ethanol produced, then ethanol was consumed as a second substrate. The glucose is the first substrate to be consumed due to the high specific growth rate this allows. Ethanol utilisation is slower growth but results in a high cell yield or MPD. In the cybernetic interpretation, the aim of a bacterium is to grow the fastest and get to a high cell number before other competitors are able to do so. A high cell yield can be achieved if a second substrate is available for cell multiplication. The aim of the bacteria growing in nutrient depleted broth is to grow fast however cell yield or MPD is limited by the lack of other nutrients available. *L. monocytogenes* grown in spent broth with no nutrient added, could be limited by nutrient depletion. Cell yield was not high in the spent broth as glucose was depleted thus another substrate remaining in the medium, was utilised to produce a high cell yield (Jones and Kompala, 1999; Patnaik, 1999; Patnaik, 2000; Patnaik, 2001; Patnaik, 2003).

Herbert and Foster (2001), reported *L. monocytogenes* to have a starvation survival mechanism when glucose is absent. In that study, a variation of the Trivett and Meyer (1971) chemically defined medium (CDM; *see* Table 3.1.1) was used to study survival of *L. monocytogenes*. Herbert and Foster (2001) found that the mechanism to help *L. monocytogenes* survive during low nutrient availability, was only activated when glucose was limiting as opposed to amino acid limitation. When amino acids were limited, *L. monocytogenes* growth ceased. Thus amino acid may not be limiting in spent broth.

When glucose is not present, other sugars can be substituted which include fructose, mannose, N-Acetylglucosamine and N-acetylmuramic acid (from bacteria cell walls), glucosamine, cellobiose, trehalose and maltose (Premaratne *et al.*, 1991). Premaratne *et al.* (1991) found growth to increase for *L. monocytogenes* by addition of chitin, cell walls of bacteria, casein/glucose and glucose alone to Modified Welshimer Broth

(MWB). Growth of *L. monocytogenes* increased by 10^7, 10^6, 10^5 and 10^4 CFU.mL^{-1} respectively. This knowledge allows us to consider the potential hazard of *L. monocytogenes* contamination on foods which may contain these sugars and other compounds including fruit (fructose), cell walls (cellobiose), malt (maltose) and vacuum packed ham (glucose).

In this study, the growth of *L. monocytogenes* in *LAB*, *E. coli* and *L. monocytogenes* spent broth, was limited by nutrient availability and not the pH of the system or the presence of waste metabolites. Therefore, nutrient limitation caused by faster growing bacteria may suppress the growth potential (MPD) of slower growing bacteria. Introducing a fast growing benign competitor (e.g. *Lb. sakei* and *Le. mesenteroides* which is naturally found on ham), to vacuum packed meats may help to prevent *L. monocytogenes* from reaching high levels, thus maintaining levels below those considered as acceptable by the European Commission (1999) (i.e. levels lower than 100 cells.g^{-1}).

Glucose appears to be the first nutrient utilised by *L. monocytogenes* however other mechanisms come into play when glucose is limited as seen with the spent broths. Glucose is present in ham (Section 1.8.2.1) therefore this information could be useful in determining the type of bacterial interactions that could occur between LAB and *L. monocytogenes*. Knowledge of the glucose content on other vacuum packed meat could be useful in predicting the type of natural inhibition that is occurring between native micro-biota and *L. monocytogenes* on meat.

Suitable competitive bacteria including non-spoilage LAB which compete for the same nutrients as *L. monocytogenes* may be inoculated onto MAP ham. *Lb. sakei* and *Le. mesenteroides* spent broth inhibited *L. monocytogenes* in this study therefore non-spoilage strains of these species could be inoculated onto MAP ham to inhibit *L. monocytogenes*. Interactions between *L. monocytogenes* and the LAB considered in this Chapter are explored further in 'in product' studies described in Chapter 5.

5 Control of *Listeria monocytogenes* by Lactic Acid Bacteria as Competitors to Growth

5.1 Introduction

The use of competitive bacteria to control *L. monocytogenes* has been suggested as a "healthier" alternative to chemical control techniques. The factors relating to the Jameson Effect (Chapter 3) are non-species specific and interactions have been observed between both non-spoilage or spoilage bacteria and pathogens, including *L. monocytogenes* (Jameson, 1962; Buchanan and Bagi, 1999). The Jameson Effect may not be observed until the MPD of spoilage bacteria is high and the food product is spoiled and non-consumable. However, some MAP RTE meats are naturally contaminated with LAB that reach stationary phase before spoilage occurs, thus causing all other bacteria, including pathogens, to stop growing (Nilsson *et al.*, 1999; Amezquita and Brashears, 2002; Ross and McMeekin, 2003).

The use of LAB as a competitive bacteria against *L. monocytogenes* has been widely reported (Winkowski and Montville, 1992; Huang *et al.*, 1993; Yang and Ray, 1994; Amezquita and Brashears, 2002; Mataragas *et al.*, 2002; Jacobsen *et al.*, 2003; Mataragas *et al.*, 2003a). LAB are considered food grade microorganisms and have been used in the past, and continue to be used today, for various food fermentations (Adams and Moss, 2000). In addition, some LAB have the following beneficial qualities:

- Pro-biotic qualities that provide health benefits for consumers of RTE meat (Nikoskelainen *et al.*, 2001; Petaja *et al.*, 2003).
- Production of bacteriocins and organic acids, which inhibit growth of other bacteria (Leroy and de Vuyst, 2001; Jacobsen *et al.*, 2003)
- Growth at refrigeration temperatures (Mataragas *et al.*, 2002).

This chapter reports on investigation of aspects of the Jameson Effect by studying interactions between LAB and *L. monocytogenes* on MAP ham.

Three *L. monocytogenes* strains isolated from smallgoods factories, the reference strain Scott A, and another food isolate were introduced as a five species cocktail to commercially produced ham products. Two different inoculum levels of the

L. monocytogenes cocktail were used to investigate the competitive advantages of higher levels in comparison to natural biota. The experiments also involved the introduction of three species of LAB that were isolated from retail ham (Chapter 3). These LAB were introduced to investigate bacterial interaction in meat with different ratios of LAB species and *L. monocytogenes*. LAB was introduced to ham to augment LAB naturally present on the product.

Bacteriocin production by LAB has been reported as the main cause of inhibition of *L. monocytogenes* (Section 1.7.2.2). This suggestion is challenged in this Chapter, by assessing the interaction of predominantly non-bacteriocin producing LAB against *L. monocytogenes*. The effect of temperature (4 and 8°C) on those interactions was also examined.

5.2 Materials and Methods

5.2.1 *L. monocytogenes* Strains

Five strains of *L. monocytogenes* were used in these experiments to allow for strain variability. The cocktail included *L. monocytogenes* (Scott A), L5/22 (isolated from smoked salmon) and three strains 20425, 20432 and 20423 from an undisclosed smallgoods factory provided by Silliker Microtech Pty. Ltd. (Appendix 1.1).

5.2.2 Lactic Acid Bacteria

The three LAB, *Leuconostoc mesenteroides, Leuconostoc carnosum* and *Lactobacillus sakei* were the most common bacteria found on the MAPham tested in this thesis (Chapter 3).

5.2.3 Challenge Study

To determine the effects of LAB on *L. monocytogenes* in processed meats, a challenge study was conducted using MAP ham as the substrate. The study involved three variables, temperature (4 or 8°C), initial concentration of added *L. monocytogenes* (10^1 or 10^3 CFU.g^{-1}) and presence or absence of added LAB. Thus there were eight treatments. Controls without added LAB or *L. monocytogenes* were also tested at each temperature. For all treatments and controls 10 triplicate samples were analysed for microbial levels over an 8-10 week period. Sampling started three days after inoculation.

Ham products were prepared and sliced by a commercial producer and ~ 50 g lots dispensed into thermoformed ridge bottom packs (PET and PE composite and produced on horizontal form fill). The packages were then filled with gas ratios of 30% CO_2:70% N_2 creating a MAP. The samples were shipped from Sydney to Hobart by commercial transport operators under refrigeration temperatures. However, no temperature records were available. Upon receipt of ham samples into this laboratory, samples were stored at 2°C for 48 h until inoculated with the test strains at the commencement of the experiment.

5.2.3.1 Inoculum Preparation

Primary inocula were prepared as follows. The five strains of *L. monocytogenes* were grown individually in TSB-Ye, containing TSB (Oxoid CM 129) and 0.6% yeast extract (Oxoid L21), incubated at 37°C for 24 h. Five colonies from each of the LAB isolate plates were placed in 10 mL MRS broths and incubated at 30°C for 24 h. *Lc. carnosum* was incubated for 48 h at 30°C because it grows more slowly (as shown in growth trials described in Chapter 4).

Both LAB and *L. monocytogenes* primary inocula were diluted in appropriate sterile broth to ~10^6 CFU.mL^{-1} (i.e. barely visible turbidity) and incubated at 10°C. Incubation at 10°C was used to simulate the physiology of cells that may arise as contaminants from the chill temperatures of a smallgoods processing facility. Growth was monitored turbidimetrically with %T readings (540nm) until inocula increased to a cell density of ~10^8 CFU.mL^{-1}.

LAB and *L. monocytogenes* cocktails of the exponential phase cultures were prepared combining 1 mL of the 10°C culture of each appropriate strain in a sterile MaCartney bottle and vortexing for one minute. Each cocktail was diluted as required in 1% peptone water with 0.85% NaCl (PW), and stored at 10°C prior to inoculation into samples.

5.2.3.2 Sample Inoculation

The LAB broth cocktail (10^1-10^2 CFU.mL^{-1}) was added to the 'high' (final density 10^3 CFU.g^{-1}) and 'low' (final density 10^1 CFU.mL^{-1}) *L. monocytogenes* cocktails in a 1:1 ratio (40 mL:40 mL). 0.1 mL of this ratio inoculum was inoculated into samples. 0.1mL of sterile diluent was added to control packets. All inocula were added *via* a syringe needle through self-adhesive rubber septa attached to the packets that were surface-sterilised with 70% ethanol before and after inoculation to prevent contamination of the pack when the inoculum was added. After addition of inocula, the septa were taped over to prevent contamination. The packets were shaken gently to disperse the introduced bacteria around the ham slices. Inoculated samples and controls were appropriately labelled and incubated at either 4 or 8°C.

5.2.3.3 *Sampling*

At appropriate intervals for each treatment triplicate samples were tested for levels of *L. monocytogenes*, LAB and total viable count. Enough samples were prepared to ensure triplicate determination of microbial loads at 10 sample times (deemed required to assess statistical significance if differences between treatments). Spread plates were prepared using a spiral plater as described in Chapter 3. Samples were taken every 2-4 d in the first 3 weeks and weekly thereafter. Sample collection was less frequent for the first four weeks for treatments incubated at 4°C on the assumption that there would be an extended lag time. At each sample time, three samples of each treatment were processed by suspending 50 g of the sample in 50 mL PW and stomaching for two minutes. As appropriate, further ten-fold serial dilutions in PW were prepared. Aliquots (0.1 mL) of the dilutions were then directly plated onto plate count agar (Oxoid CM 643; PCA), PALCAM (Oxoid CM877 and SR150 supplement; Listeria selective agar) and MRS agar (Oxoid CM361; MRS). PALCAM plates were used to enumerate *Listeria* spp. and MRS was used to enumerate LAB. The PCA was used to determine total viable (aerobic) count. PALCAM plates were incubated for 48 h at 37°C while MRS and PCA plates were incubated for 72 h at 20°C.

Enumeration of *L. monocytogenes* levels <10 $CFU.g^{-1}$ was achieved by plating 4 x 250 µL of the 1:2 dilution on quadruplicate plates. This allowed detection of >2 $CFU.g^{-1}$ which may be a realistic level of *L. monocytogenes* on processed meats (Norrung *et al.*, 1999; Ross *et al.*, 2004).

5.2.4 Analysis of Data

Generation times were calculated by plotting $\log_{10}CFU.g^{-1}$ for total viable count (TVC), *L. monocytogenes* and LAB against time. MPD was calculated by averaging either 3 or 4 of the highest points reached by the bacteria. The generation time (GT) was calculated from the gradient of the linear regression fitted to the exponential phase of the growth curves, i.e. GT was calculated by dividing 0.301 by the gradient. Growth rate was calculated as the reciprocal of the GT estimate (see Chapter 3).

5.2.5 $T_{equivalent}$ Values

$T_{equivalent}$ is the temperature beyond which one bacterial strain grows faster than another strain for a specific set of environmental conditions (J. Olley, *pers. comm.*). For any two organisms it can be determined from Equation 5.1 (T. Ross, *pers comm.*, 2004):

$$T_{eq} = (b_1 T_{min1}) - (b_2 T_{min2}) / (b_1 - b_2) \qquad \text{Equation 5.1}$$

where $T_{equivalent}$ is the "cross over" temperature and b1 and b2 are the coefficients of the square root equations (see Equation 5.2) describing the growth of the two organisms considered.

The square root model (Ratkowsky *et al.*, 1982) is:

$$R = b(\text{Temperature} - T_{min})^2 \qquad \text{Equation 5.2}$$

Where R is the relative growth rate (usually calculated as 1/GT), b is the gradient, "Temperature" is the actual incubation temperature and T_{min} is the theoretical minimum temperature for growth estimated as the temperature at which $R = 0$ (i.e. the x- intercept of a plot of $\sqrt{R}$ against temperature). The gradient is calculated by linear regression of $\sqrt{(R)}$ *vs.* temperature.

$T_{equivalent}$ was determined for *L. monocytogenes* in co-culture with *Lc. carnosum*, *Lb. sakei* and *Le. mesenteroides*. The $T_{equivalent}$ was derived to determine under what conditions in MAP ham *L. monocytogenes* growth might be expected to be inhibited by LAB. For calculation, the T_{min} values taken were those presented in Table 3.3 which were for growth in environments simulating MAP ham products.
Those conditions are as follows:

Water activity: 0.970 (From Chapter 2, smallgoods water activity results)
pH: 6.2 (From Chapter 2 smallgoods pH results)
Lactic acid: 225mg/50g (Devlieghere *et al.*, 2001)
Nitrite: 0

The gradient (b value) for *Le. mesenteroides*, *Lc. carnosum* and *Lb. sakei* was calculated from growth curve data at 20 and 30°C (Chapter 3). The growth rates of

the endogenous LAB at 4 and 8°C were calculated from data presented in this Chapter (Figures 5.11 – 5.13). The known b and T_{min} values were substituted into Equation 5.1 to calculate $T_{equivalent}$ values.

5.3 Results

5.3.1 Initial Levels, MPD and Growth Rates of *L. monocytogenes* and LAB

L. monocytogenes inocula levels added to treatments are shown in Table 5.1. The 'low' *L. monocytogenes* inoculum was close to 10 CFU.g^{-1} whereas the 'high' inoculum level was close to 10^3 CFU.g^{-1}.

Table 5.1 Levels of *L. monocytogenes* added to samples.

Inoculum type	Inoculum level achieved (CFU.g^{-1})
High *L. monocytogenes* (HL)	1.2 x 10^3
Low *L. monocytogenes* (LL)	1.1 x 10^1

The initial inoculum level of native ('endogenous') LAB already found on meat, was close to 100 CFU.g^{-1} whereas the total LAB level after exogenous LAB were added, was close to 1000 CFU.g^{-1} (Table 5.2). Therefore, the numbers of LAB added to MAP ham samples was estimated to be ~900 CFU.g^{-1}.

Table 5.2 The average initial level of LAB on all treatments inoculated with *L. monocytogenes*.

Initial level (CFU.g^{-1})	4 °C		8°C	
	HL	LL	HL	LL
Endogenous LAB only	$1.10x10^3$	$2.02x10^2$	$2.25x10^3$	$2.06x10^2$
Endogenous + Added LAB	$8.47x10^2$	$1.09x10^3$	$1.14x10^4$	$1.09x10^3$

Microbiological changes in the various treatments as a function of incubation time are shown in Figures 5.1 to 5.8. In all figures, the time axis represents days since the

product was packed. Products were inoculated 11 days after initial packaging. Thus, time = 11 days represent time = 0 for the experiment.

At 8°C, *L. monocytogenes* reached a MPD of 2 logs greater than the initial inoculum for either level of 10^1 and 10^3 CFU.g^{-1} (Figures 5.1 and 5.2). MPD was achieved at around day 25 to 30 when the MPD for TVC and introduced LAB reached close to 9 log CFU.g^{-1}.

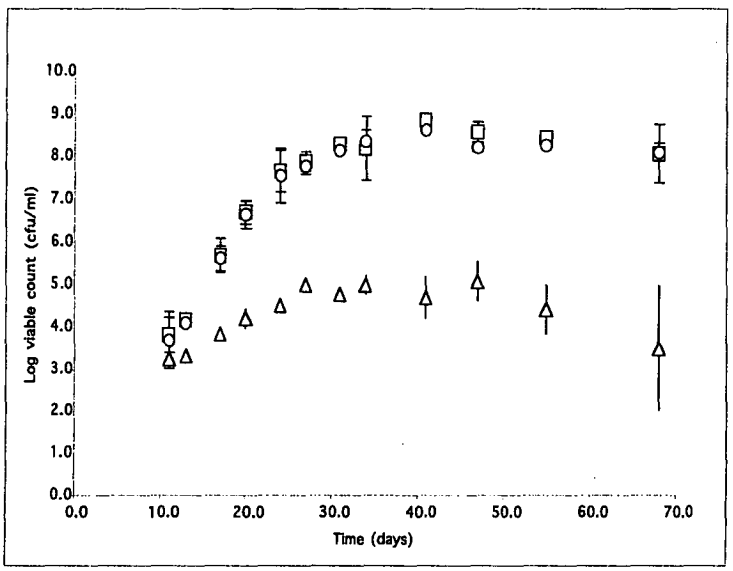


Figure 5.1 Growth of lactic acid bacteria and *Listeria monocytogenes* on MAP ham at 8 °C with a 'high' initial level of *L. monocytogenes*. (Δ): *L. monocytogenes* ; (○): lactic acid bacteria ;(❑): the total viable count.

At 4 °C, no *L. monocytogenes* growth was observed for inoculum levels of 10^1 and 10^3 CFU.ml^{-1} with added LAB[1]. The added LAB and native ham MAP bacteria reached a MPD of around 8 log CFU.g^{-1} (Figure 5.3 and 5.4).

[1] Error bars- three representative samples within an experiment.

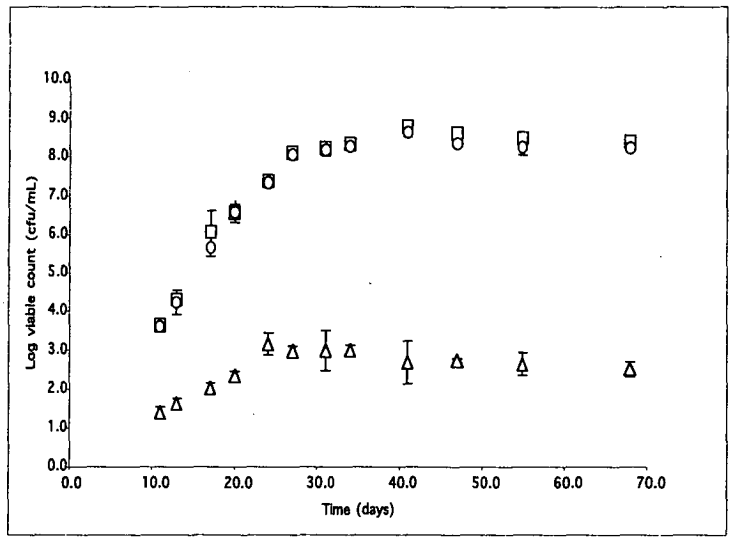


Figure 5.2 Growth of lactic acid bacteria and *Listeria monocytogenes* on MAP ham at 8 °C with a 'low' initial level of *L. monocytogenes*. (Δ): *L. monocytogenes* ; (○): lactic acid bacteria ;(❑): the total viable count.

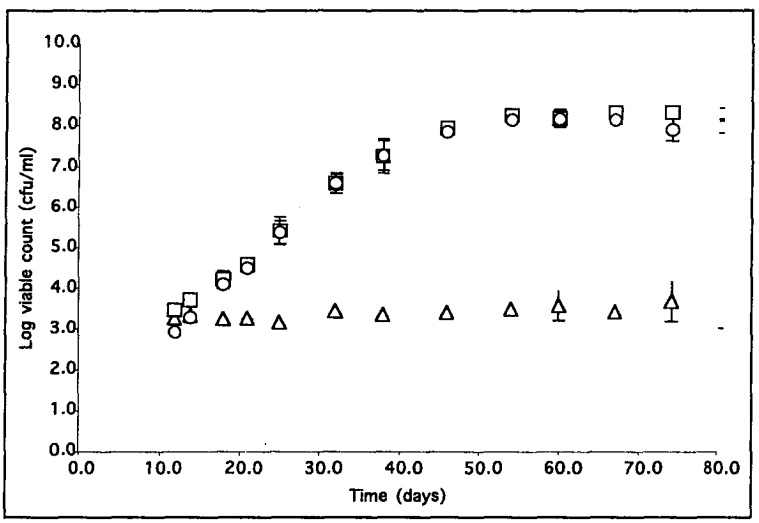


Figure 5.3 Growth of LAB and *Listeria monocytogenes* cocktail inoculum levels of 10^3 CFU.g^{-1} introduced to MAP ham at 4°C where (Δ) is *L. monocytogenes*, (○) is total lactic acid bacteria and (❑) is the total viable count and ~10^3 CFU.g^{-1} of a three strain LAB cocktail was also added at the commencement of the experiment.

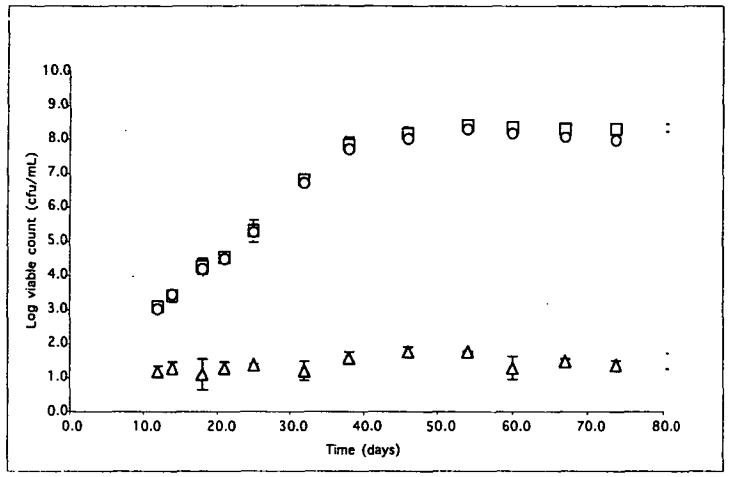


Figure 5.4 Growth of LAB and *Listeria monocytogenes* cocktail inoculum levels of 10^1 CFU.g^{-1} introduced to MAP ham at 4 °C where (Δ) is *L. monocytogenes*), (O) is total lactic acid bacteria and (□) is the total viable count and ~10^3 CFU.g^{-1} of a three strain LAB cocktail was also added at the commencement of the experiment.

The *L. monocytogenes* cocktail grew in MAP ham at both 4 and 8°C in samples to which no LAB were added, and at 8°C but not at 4°C in treatments with added LAB. Greater variations between replicates were also observed without added LAB (see error bars in Figures 5.5 and 5.6 compared to Figures 5.1 and 5.2).

In all cases, LAB grew faster than *L. monocytogenes* at 4°C and 8°C. The MPD of *L. monocytogenes* was reduced from 10^7 CFU.g^{-1} to 10^5 CFU.g^{-1} in samples held at 8°C to which the LAB cocktail was inoculated (Table 5.3). Other MPD reductions were observed when added LAB were present. This was expected to occur as a consequence of faster growth rate of the LAB and the stationary phase of the LAB being reached more quickly when added LAB were present. MPD reached by LAB in the control samples, and when LAB was added to ham, are summarised in Table 5.3.

Table 5.3 MPD achieved by *L. monocytogenes* in the presence of endogenous LAB only and endogenous LAB + added LAB in MAP ham

L. monocytogenes (CFU.g^{-1})	Endogenous LAB (CFU.g^{-1})		Endogenous + Added LAB (CFU.g^{-1})	
	4 °C	8 °C	4 °C	8 °C
~10^3	10^4	10^7	10^3	10^5
~10^1	10^2	10^4	10^1	10^3

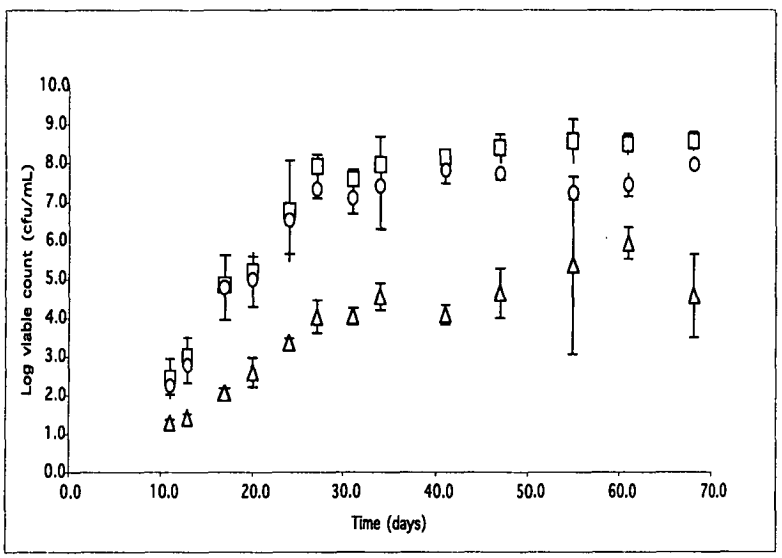


Figure 5.5 Growth of endogenous LAB (control) and *Listeria monocytogenes* inoculum levels of 10^1 CFU.g^{-1} on MAP ham at 8 °C where (Δ) is *L. monocytogenes*, (○) is total lactic acid bacteria and (❑) is the total viable count.

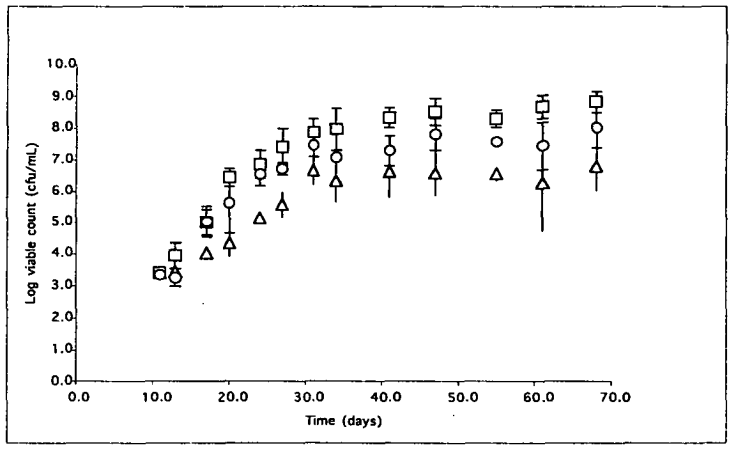


Figure 5.6 Growth of native LAB (control) and *Listeria monocytogenes* inoculum levels of 10^3 CFU.g^{-1} on MAP ham at 8 °C where (Δ) is *L. monocytogenes*, (○) is total lactic acid bacteria and (❑) is the total viable count

The treatments with no added LAB at 4°C showed less growth of *L. monocytogenes* than at 8°C, where the MPD increased by only ~1 log CFU.g^{-1} (see Figure 5.7 and 5.8) for both inoculum levels. Endogenous LAB and other bacteria reached a MPD of around 8 log CFU.g^{-1}.

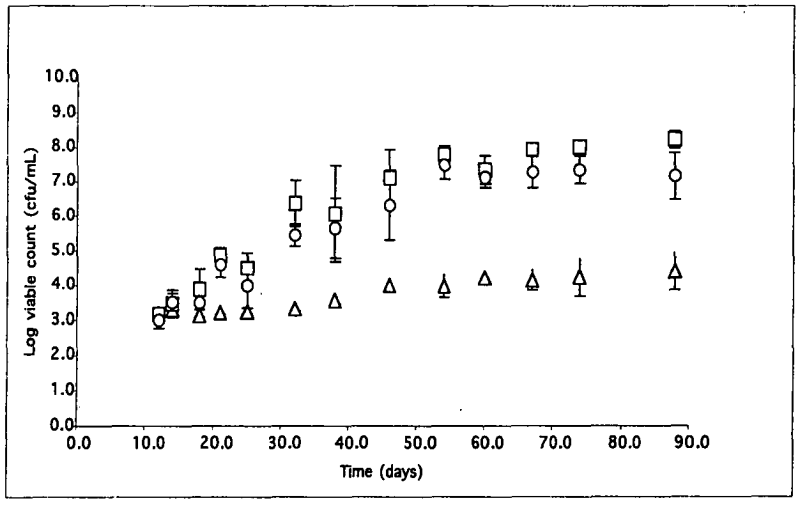


Figure 5.7 Growth of native MAP ham LAB (control) and *Listeria monocytogenes* inoculum levels of 10^3 CFU.g^{-1} on MAP ham at 4 °C where (Δ) is *L. monocytogenes*, (○) is total lactic acid bacteria and (❑) is the total viable count.

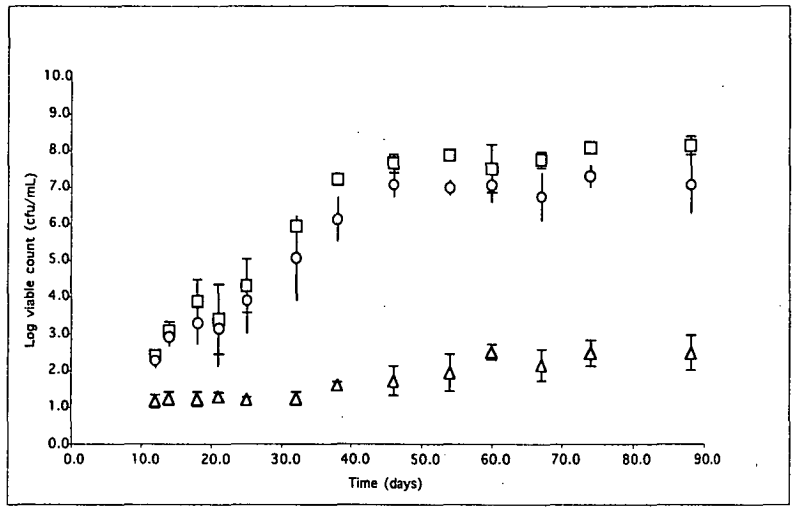


Figure 5.8 Growth of native MAP ham LAB (control) and *Listeria monocytogenes* inoculum levels of 10^1 CFU.g^{-1} on MAP ham at 4 °C where (Δ) is *L. monocytogenes*, (○) is total lactic acid bacteria and (❑) is the total viable count.

The MPD reached for LAB at 4°C and 8°C was between 8 and 9 log CFU.g^{-1} for all treatments regardless of the temperature or starting inoculum level (Figure 5.9 and 5.10). The MPD level reached by LAB in the presence of naturally occurring LAB and added LAB is recorded in Table 5.4. The added LAB on MAP ham achieved an extra 'log' of growth compared to the naturally occurring LAB on the ham.

Table 5.4 The average MPD reached by total LAB in the presence of added LAB and *L. monocytogenes*.

Initial *L. monocytogenes* Level	MPD achieved (cfu.g^{-1})	
	8°C	4 °C
Added LAB		
10^3 CFU.mL^{-1}	2.7 x 10^8	1.42 x 10^8
10^1 CFU.mL^{-1}	2.93 x 10^8	1.53 x 10^8
No Added LAB		
Control (no Listeria)	1.5 x 10^7	2.7 x 10^7

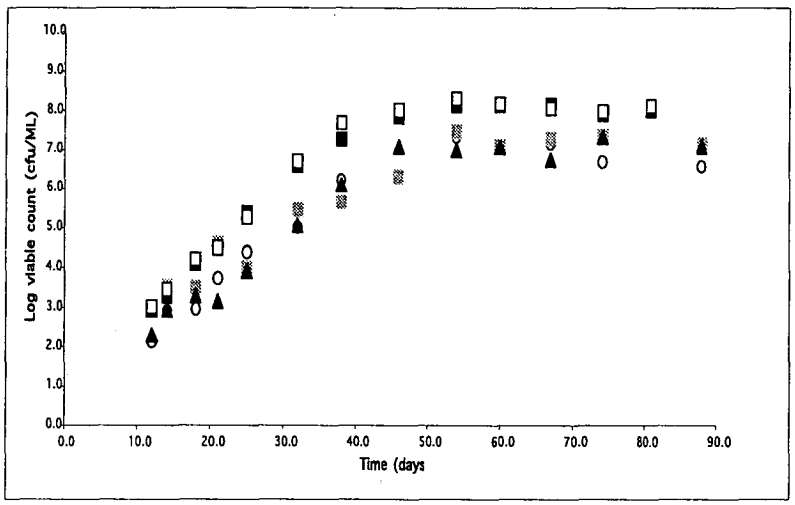


Figure 5.9 The log viable count ($CFU.g^{-1}$) of LAB on all ham treatments stored at 4°C, where (❍) is the Control sample, (▩) is the treatment where 10^3 $CFU.g^{-1}$ *L. monocytogenes* was inoculated on to the control, (▲) is the treatment where 10^1 $CFU.g^{-1}$ of *L. monocytogenes* was inoculated onto the control, (■) is 10^3 $CFU.g^{-1}$ *L. monocytogenes* and LAB inoculated onto ham and (❑) is 10^1 $CFU.g^{-1}$ of *L. monocytogenes* and LAB added to ham.

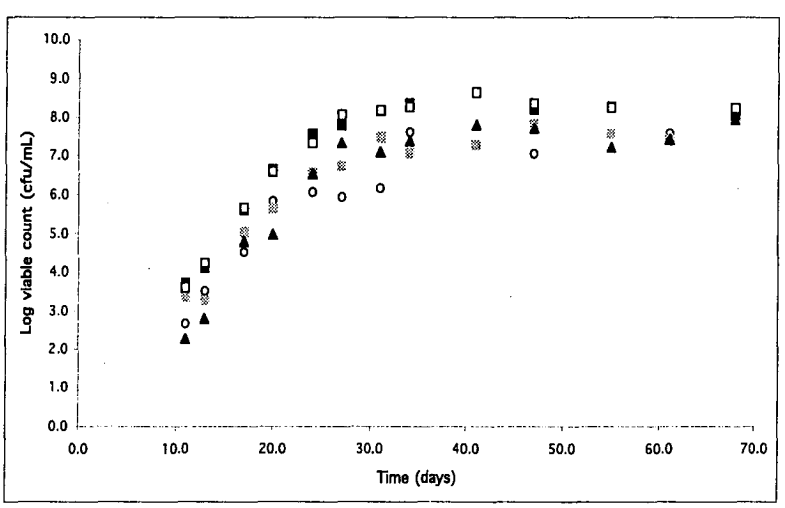


Figure 5.10 The log viable count ($CFU.g^{-1}$) of LAB on all ham treatments stored at 8°C, where (❍) is the Control sample, (▩) is the treatment where 10^3 $CFU.g^{-1}$ *L. monocytogenes* was inoculated on to the control, (▲) is the treatment where 10^1 $CFU.g^{-1}$ of *L. monocytogenes* was inoculated onto the control, (■) is 10^3 $CFU.g^{-1}$ *L. monocytogenes* and LAB inoculated onto ham and (❑) is 10^1 $CFU.g^{-1}$ of *L. monocytogenes* and LAB added to ham.

The combined viable count of LAB and *L. monocytogenes* was less than the TVC for the *L. monocytogenes* inoculum samples, thus other micro-organisms were present on ham. Figures 5.1 to 5.4 show smaller variations between TVC and LAB count compared with Figures 5.5 to 5.8 which describe the microbiological changes in the analogous samples to which no exogenous LAB were added. These differences may demonstrate the dominant growth characteristics of the introduced LAB.

The growth rate was faster for LAB grown at 8°C compared to *L. monocytogenes'* growth rate (Tables 5.5a, b and 5.6). This observation was similar for growth at 4°C. These growth data were used to calculate the $T_{equivalent}$ value.

Table 5.5a Generation time (GT), and growth rate (GR) of *L. monocytogenes* and LAB in treatments with added LAB + endogenous LAB at 4 and 8°C.

	4 °C				8 °C			
Initial *L. monocytogenes* level	10^1 CFU.g^{-1}		10^3 CFU.g^{-1}		10^1 CFU.g^{-1}		10^3 CFU.g^{-1}	
	Listeria	LAB	Listeria	LAB	Listeria	LAB	Listeria	LAB
GT (day)	-	1.68	-	1.75	2.30	1.10	2.90	1.11
GR (gens. day^{-1})	-	0.59	-	0.57	0.43	0.90	0.34	0.90

Table 5.5b Generation Time (GT), growth rate (GR) of *L. monocytogenes* and LAB in treatments with no added LAB (endogenous LAB) at 4 and 8°C.

	4 °C				8 °C			
Initial *L. monocytogenes* level	10^1 CFU.g^{-1}		10^3 CFU.g^{-1}		10^1 CFU.g^{-1}		10^3 CFU.g^{-1}	
	Listeria	LAB	Listeria	LAB	Listeria	LAB	Listeria	LAB
GT (day)	7.6	2.16	11.2	2.8	1.60	0.95	1.90	1.38
GR (gens.day^{-1})	0.132	0.526	0.089	0.357	0.625	1.111	0.526	0.555

Table 5.6 Generation Time (GT), Growth Rate (GR) and Lag time of endogenous LAB with no added LAB or *L. monocytogenes*.

Parameters (days)	4 °C	8 °C
GT	2.4	1.1
GR	0.416	0.909
Lag time	9.0	8.3

5.3.2 $T_{equivalent}$ Values for LAB versus *L. monocytogenes*

$T_{equivalent}$ temperatures for *L. monocytogenes* versus *Le. mesenteroides*, *Lc. carnosum* and *Lb. sakei* and LAB grown on MAP ham or in environments analogous to ham, are shown in Table 5.7. LAB grew faster than *L. monocytogenes* in a MAP ham environment at temperatures less than those listed below. The square root plots for LAB and *L. monocytogenes* are displayed as Figures 5.11 to 5.13. The $T_{equivalent}$ values are where the lines of best fit cross each other. These values are shown in Table 5.7 where the shaded area highlights the similarity between $T_{equivalent}$ values for *Lb. sakei* and the endogenous LAB on MAP ham.

Table 5.7 The $T_{equivalent}$ temperatures for LAB against *L. monocytogenes* under environmental conditions analogous to MAP ham (*viz.* pH 6.5 and a_w 0.975)

Source and conditions	*Le. mesenteroides* (°C)	*Lb. sakei* (°C)	*Lc. carnosum* (°C)	Endogenous LAB (°C)	Endogenous LAB + Added LAB (°C)
Soontranon, 1998; pH 5.5, a_w 0.975, GT (mins): 7440 (4°C) 1440 (8°C)	11.5✞	14.0*	-3.8✞	-	-
Soontranon, 1998; pH 6.5, a_w 0.975, GT (mins): 5424 (4°C) 1038 (8°C)	-	-	-	5.4*	6.40*
Ham trial; inoculum level 10^1 CFU.g^{-1}, GT (mins): 10944 (4°C) 2304 (8°C)	-	-	-	13.0*	10.0*
Ham trial; inoculum level 10^3 CFU.g^{-1}, GT (mins): 16128 (4°C) 2736 (8°C)	-	-	-	15.1*	12.0*

* LAB faster than *L. monocytogenes* up to $T_{equivalent}$.

✞ *L. monocytogenes* faster than LAB up to $T_{equivalent}$.

shaded area highlights similarity between $T_{equivalent}$ for *Lb. sakei* endogenous LAB

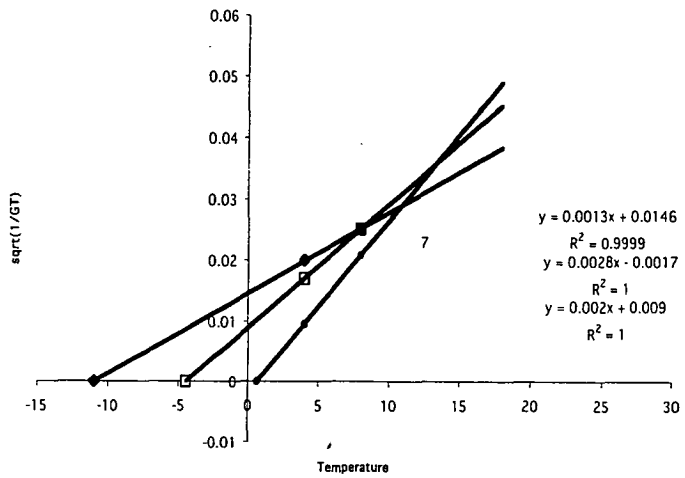


Figure 5.11 √(1/GT) versus temperature plot where (◆) is endogenous LAB, (❑) endogenous LAB + added LAB and (●) is *L. monocytogenes* inoculated at a level of 10^1 CFU.g^{-1} on ham at 4 and 8°C.

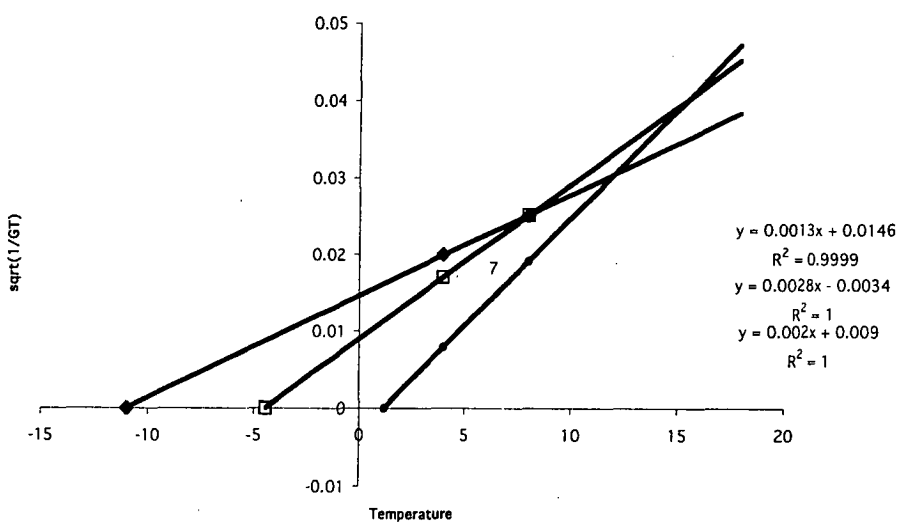


Figure 5.12 √(1/GT) versus temperature plot where (◆) is endogenous LAB, (❑) endogenous LAB + added LAB and (●) is *L. monocytogenes* inoculated at a level of 10^3 CFU.g^{-1} on ham at 4 and 8°C.

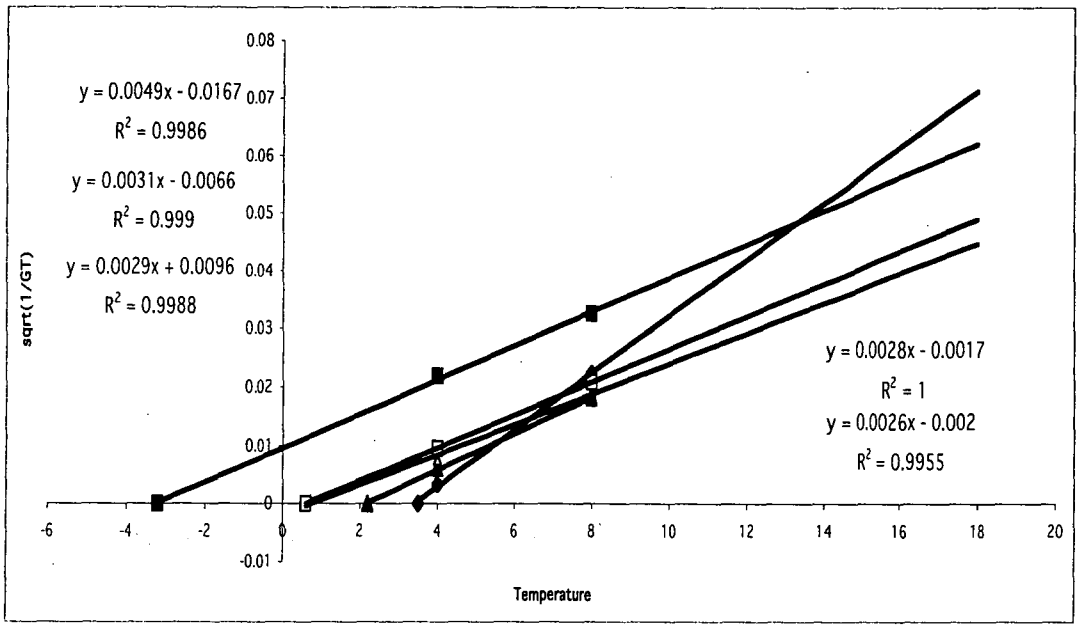


Figure 5.13 $\sqrt{(1/GT)}$ versus temperature plot where (◆) is *Le. mesenteroides*, (▲) is *Lc. carnosum* , (■) is *Lb. sakei* growing at 4 and 8°C in MRS broth, pH 5.5, a_w 0.970. The growth rates of the LAB were compared to *L. monocytogenes* growing on MAP ham, which included (❑) *L. monocytogenes* (10^1 CFU.g^{-1}) and (Δ) *L. monocytogenes* (10^3 CFU.g^{-1}). The T_{min} value used for LAB was taken from Chapter 3, Table 3.3. The T_{min} value for *L. monocytogenes* of 0.57°C is derived from an unpublished model for *L. monocytogenes* growth rate (Ross, *pers. comm.*, 2004).

5.4 Discussion

The introduction of *Lb. sakei*, *Le. mesenteroides* and *Lc. carnosum* to commercial MAP ham products inhibited the growth of *L. monocytogenes*. Different levels of *L. monocytogenes* inhibition were seen at 8°C when *L. monocytogenes* was inoculated onto meat at a level of 10^3 CFU.g^{-1} and 10^1 CFU.g^{-1}. The difference in level of *L. monocytogenes* MPD at 4°C with or without added LAB was only 0.5 to 1 logs compared to storage at 8°C, where addition of LAB restricted growth to 2 logs compared to 3.5 logs with endogenous LAB only. At 4°C, the addition of LAB reduced the *L. monocytogenes* MPD to10^3 CFU.g^{-1} (*cf.* endogenous LAB that limited "high" *L. monocytogenes* inocula to an MPD of 10^4CFU.g^{-1}). The MPD reached by *L. monocytogenes* initially inoculated onto meat at a level of 10^3 CFU.g^{-1}, with endogenous LAB or added LAB, was 10^7 and 10^5 CFU.g^{-1} respectively (Table 5.3). A similar pattern of inhibition was noted with the lower *L. monocytogenes* inoculum (10^1 CFU.g^{-1}) where the final MPD difference between added LAB and endogenous LAB was 1 log (i.e. 10^4 *cf.* 10^3 CFU.g^{-1} with added LAB). Therefore, the level of MPD reached by *L. monocytogenes* was affected by its initial inoculum level, temperature of the product and initial level of other LAB. It is likely that the inhibition of *L. monocytogenes* was due to faster growth rate of the native LAB and/or added LAB compared to *L. monocytogenes* such that they reached stationary phase first and imposed a Jameson Effect. *Lb. sakei*, in particular, had a faster growth rate and lower temperature growth limits than *L. monocytogenes* (Table 5.7).

AFSSA (2001; *cited* in Uyttendaele *et al.*, 2004) recommends an inoculum level of 10^3 CFU.g^{-1} for challenge testing. This level was challenged by Uyttendaele *et al.* (2004) who conducted challenge tests which included a *L. monocytogenes* inoculum level of 10^2 CFU.portion^{-1} (i.e. 1-10 cells.g^{-1}) as 10^3 CFU.g^{-1} is not a realistic level found on RTE meats. Krockel (2000) conducted a survey on sliced pre-packed RTE meats and found the positive samples for *L. monocytogenes* contained no more than 10^2 CFU.g^{-1} *L. monocytogenes* at the end of the nominated shelf life of the products. In this study, *L. monocytogenes* inoculated onto MAP ham at a level of 10^1 and 10^3 CFU.g^{-1} experienced a long lag phase when incubated at 4°C. However, at 8°C no significant lag phase was observed.

The average MPD reached by introduced LAB at 4 and 8°C was approximately 10^8 CFU.g^{-1} (Table 5.4). However, the final MPD for LAB already present on the product, was approximately 10^7 CFU.g^{-1}. In those samples, however, the total viable count exceeded 10^8 CFU.g^{-1} suggesting that the 'non-LAB' bacteria in the ham induced a Jameson Effect on the endogenous LAB. Addition of LAB increased the total LAB present on ham from a MPD of 10^7 CFU.g^{-1} to a MPD of 10^8 CFU.g^{-1} sufficient for the LAB to dominate the microbial population and induce a Jameson Effect on the other microbiota. A similar MPD for LAB was also reported by Krockel (2000) who observed that the LAB load exceeded 10^8 CFU.g^{-1} for the majority of RTE meats in that study. In the current study, the increased growth of LAB after addition of LAB, may be explained by the fact that LAB were added to ham at the exponential growth stage and/or *Lb. sakei* dominated the other bacteria by virtue of its faster growth rate. Other microbes dominated when LAB was not introduced (Section 5.3.1).

Inhibition of one bacterial strain by another has been related to bacteriocin production as discussed in Section 1.6. Other studies have suggested that inhibition of MPD is due to non-specific suppression of all other bacteria when the fastest bacteria reach MPD, the Jameson Effect (Section 1.6.3). The faster growing bacteria will determine the level of MPD for all other bacteria in the system (Patnaik, 1999; Ross and McMeekin, 2003) and the rate and extent of growth of bacteria is affected by nutrient availability and environmental factors (i.e. pH, temperature and organic acids). Thus, this knowledge is instrumental in examining aspects of the Jameson Effect. The Jameson Effect was observed clearly in all treatments stored at 8°C and in treatments at 4°C with no added LAB. It is probable, also, that the Jameson Effect was induced by the LAB in the 4°C treatment with added LAB but that the effect was induced prior to the introduced *L. monocytogenes* population emerging from its lag phase. Individual *L. monocytogenes* strains were tested against the three LAB strains for possible bacteriocin production by those LAB (*see* Chapter 3). Two strains of *L. monocytogenes* were inhibited by two strains of LAB, therefore, some inhibition of *L. monocytogenes* may have been caused by certain strains producing bacteriocin. However, three strains were not susceptible to bacteriocin production and would be expected not to be inhibited by this mechanism, yet inhibition of the entire *L. monocytogenes* population, comprising five strains, was observed.

Consequently, other mechanisms must be responsible for the inhibition of *L. monocytogenes* growth that was observed.

LAB reached MPD between 20-30 d at 8°C. The time required for *L. monocytogenes* to reach MPD appears to be determined by the LAB (the first bacteria to reach MPD), thus *L. monocytogenes* also reached MPD between 20-30 d but at a lower level (Figures 5.1-5.8). *L. monocytogenes* had a slower growth rate than LAB at both temperatures tested and, as a consequence, the MPD reached for *L. monocytogenes* was 10^3 and 10^5 CFU.g^{-1} for 'low' and 'high' levels of *L. monocytogenes* respectively. LAB grew faster than *L. monocytogenes* at 4 and 8°C thus enabling LAB to reach maximum population before *L. monocytogenes*. Tamplin (2004, *unpublished*) conducted a trial in which a cocktail of *L. monocytogenes* strains (H7776, HAT-2, JBL2365, JF, 101M and F2365) were inoculated onto sterile irradiated ham (i.e. no endogenous micro-organisms present) and incubated at 5 and 10 °C. The growth rate for *L. monocytogenes* on irradiated meat in Tamplin's study was almost twice as fast as the *L. monocytogenes* growing on MAP RTE ham in this study. In Tamplin's study, the growth rate of *L. monocytogenes* at 10°C and 5°C was 0.796 and 0.702 generations.day^{-1} respectively. In this study the growth rate of *L. monocytogenes* at 8°C was 0.434 generations.day^{-1} (high inoculum) and 0.344 generations.day^{-1} (low inoculum). *L. monocytogenes* growth rate was slower than LAB growth rate at 4°C (summarised in Table 5.5). These observations and other reports (Farber *et al.*, 1996; Duffes *et al.*, 1999; Bedie *et al.*, 2001) suggest Tamplin's (2004, unpublished) results may be anomalous. Therefore the formulation of the ham or the types and numbers of LAB present may be inhibiting the growth of *L. monocytogenes*.

Most storage temperatures are rarely constant at 4°C as shown in Table 5.8 (Ross *et al.*, 2004). For example the temperatures at retail storage on average are several degrees above the recommended 4°C. However, if enforced in the smallgoods/retail food industry, 4°C storage would appear to greatly reduce the risks from listeriosis because this temperature alone appeared to inhibit *L. monocytogenes* growth in this study. However, inhibition of *L. monocytogenes* at 4°C is debatable because some researchers support this finding (Grau and Vanderlinde, 1992; Buchanan and Bagi, 1999; Coleman *et al.*, 2003) whereas other models have shown growth of *L. monocytogenes* at 4°C (Ross *et al.*, 2004).

Table 5.8 Summary of temperatures in the Australian meat cold chain, including consumers (*Reproduced from* Ross *et al.*, 2004).

Section of Chain	Average Temperature (°C)	
	Winter	Summer
Processor to Retail	5.5 (11.9)*	4.3 (11.7)
Retail Distribution Centre	2 – 4 (based on expert opinion)	
Retail Storage (USA data)		
pre-packed processed meat	6.4 (18.9)	
sliced at store	7.1 (17.8)	
Retail Display Cabinets (Australia)		
average of four types	6.0 (9.7)	
Consumer Transport	10.3 (15.3)	19.8 (27.8)
Australian Domestic Refrigerators	3.7 (9.3)	4.0 (9.2)
Australian Catering	3.6 (6.9)	4.2 (10.4)

* values in brackets indicate the maximum temperature (°C) recorded.

Predictive models for microbial growth can be utilised to compare actual data with estimates derived from the model data extracted from the model (McMeekin *et al.*, 1993). In this study the average a_w for ham stored at 4 and 8°C, was 0.970 and 0.967 respectively and pH ranged from 6.0 to 6.5. These pH and a_w values were substituted into the *L. monocytogenes* growth rate model to estimate the growth rate of *L. monocytogenes* at 4°C (Ross, *pers. comm.*, 2004). The predicted growth rate for *L. monocytogenes* was 0.20 generations.day^{-1} at 4°C for either a_w (assuming pH = 6.25, lactic acid concentration = 30 mM, nitrite = 25ppm). The absence and/or slow growth of *L. monocytogenes* at 4°C on MAP ham in the experiments described here may have been due to an extended lag time or other constraints or constituents in the MAP ham (eg. higher lactate or nitrite levels). On the other hand, growth rate for *L. monocytogenes* at 8°C was predicted to be 0.93 generations per day (assuming pH = 6.25, lactic acid concentration = 30 mM, nitrite = 25ppm). The observed growth rates for high and low *L. monocytogenes* inoculum were 0.43 and 0.34 generations.day^{-1} respectively. The predicted rate is more than double that observed found in this study

From Table 5.8 it can be seen that it is unrealistic to assume that temperatures within smallgoods distribution and sale are maintained at 4°C, thus other control strategies are required to inhibit *L. monocytogenes* growth. Non-chemical control treatments

are desirable in the food industry from a marketing and food regulation stand point. Microbial competition and suppression of pathogen growth may be a "greener" or "healthier" alternative to chemical treatments (Section 1.7).

Irradiation, high pressure processing (HPP) and addition of salts of lactate and diacetate have been employed to suppress growth of, or to eliminate, all microbes on the meat surface. The impact of pathogen growth in the absence of endogenous microbes on meat is important to consider. Further research relating to this thesis could involve inoculating *L. monocytogenes* directly onto the surface of a sterile piece of meat to observe the growth of *L. monocytogenes* on a meat medium without the endogenous microbial population. In Tamplin (2004)'s study, the average MPD for the *L. monocytogenes* cocktail was 9.66 logs (4.5×10^9 CFU.g^{-1}) and 9.70 logs (4.9×10^9 CFU.g^{-1}) for 10 and 5°C respectively. These MPD levels are significantly higher than the MPD levels reached by *L. monocytogenes* (i.e. between 10^7 to 10^1 CFU.g^{-1}) in this study with the presence of endogenous LAB and added LAB. In summary, the results of the current study suggest that the MPD of *L. monocytogenes* was suppressed by ≥2 logs by endogenous microbiota present on the ham and as high as > 4 logs by additional LAB added to ham at 8°C.

The $T_{equivalent}$ concept was introduced as means to calculate the temperature at which one bacterium grows at a faster rate than another (Section 5.2.5). *Lb. sakei* will grow faster than *L. monocytogenes* on MAP ham until the temperature is above 10°C (Table 5.7). Similar $T_{equivalent}$ temperatures (i.e. 10 to 15°C) were estimated for endogenous LAB and added LAB against *L. monocytogenes*. "Endogenous LAB" and "Added LAB + endogenous LAB" had similar $T_{equivalent}$. The dominant LAB present in the endogenous micro-organisms may have been *Lb. sakei* as its $T_{equivalent}$ temperature is similar to that for the endogenous LAB. *Lb. sakei* present in the added LAB may have also have become the pre-dominant species. The T_{min} of "Endogenous LAB" and "Added LAB" were –4.45 and –11.0°C respectively, and were estimated from two temperature points available from the ham trial. The low T_{min} value of the 'endogenous' LAB suggest *Lb. sakei* may have been the dominant LAB in the bacterial race. These comments based on $T_{equivalent}$ calculations are speculative as few data were available, resulting in extrapolations undertaken with only 3 points. Collection of more data, for example at 5 degree temperature intervals, would allow more accurate calculations and more informed comments.

Similar observations were reported by Grau and Vanderlinde (1992) where at 0°C *L. monocytogenes* grew at about half the rate of other flora (i.e. LAB and *B. thermosphacta*) on corned-beef. However at 9°C both groups of organisms grew at the same rate. It is useful to know the temperature at which LAB will become inhibitory as some products may experience temperature abuse. For example, meat transported by consumers in their own cars was reported to be as high as 27.8°C (Table 5.8). This temperature would fall outside the range of the Jameson Effect (10 to 15°C) thus *L. monocytogenes* may be able to grow faster than the bio-control LAB i.e. the Jameson effect will only be reliable at temperatures below 10 - 15°C.

Developing nations may not have access to, or employ, advanced pathogen control technologies (i.e. including control techniques mentioned above and HACCP). However these countries may have natural micro-biota limiting the growth of pathogens on some foods. This can be demonstrated in unpasteurised cheeses and cheeses with added starter cultures (Giraffa *et al.*, 1994; Rodriguez *et al.*, 2001). Although these suggestions are speculative, the relevant question to ask would be "Is the incidence of *L. monocytogenes* significantly higher in third world countries compared to the western world?" and "Are the endogenous bacteria causing the Jameson Effect thus suppressing the *L. monocytogenes*?" .

There were large variations in viable counts in some triplicate samples as indicated by the error bars seen in Figures 5.5 to 5.8. This may be due to random selection of samples from the slicer machine for each treatment. For example, samples going through the slicer at an earlier stage of production might have less bacterial contamination compared to samples going through at a later stage of production. Slicers have been associated with contamination by pathogens and LAB (Section 1.4). Larger variances were noted in the control samples where LAB had not been introduced. This would be expected due to the consistent level of added LAB nullifying the variability in initial endogenous LAB levels (see Table 5.2). Introduction of LAB created less variation possibly due to the dominant nature (i.e. faster growth rate) of the *Lb. sakei* introduced. Such variability could be better assessed by recording for each sample the sequence in which it passes through the slicer.

While 4°C is the preferred temperature for ham storage, this temperature currently can not be assumed to be maintained throughout the smallgoods 'cold chain' (Table 5.8). Therefore, the results obtained from the challenge studies at 8°C storage may be more relevant to industry. From the results the *L. monocytogenes* MPD achieved depends both on the starting number of *L. monocytogenes*, and the starting number of LAB or other bacteria on the product, and the storage temperature. At 8°C storage, when the starting number of LAB was increased with added LAB, the *L. monocytogenes* MPD dropped from 10^7 to 10^5 CFU.g^{-1}. In addition to this, a lower initial inoculum level of *L. monocytogenes* and added LAB resulted in the MPD of *L. monocytogenes* dropping from 10^4 to 10^3 CFU.g^{-1}. Food products with *L. monocytogenes* levels above 10^2 CFU.g^{-1} are considered to pose an unacceptable risk of listeriosis for consumption (European Commission, 1999; CAC, 2002).

One hundred cells of *L. monocytogenes* falls below the 10th percentile of the infective dose range for the YOPI population, 10^5-10^7 CFU predicted by Faber *et al.* (1996) (Section 1.6.2). The infective dose for the "healthy" population outside this group was estimated to be 10^7-10^9 CFU (Farber *et al.*, 1996). WHO/FAO (2004) concluded that even levels as high as 1000 CFU.g^{-1} at the point of consumption were unlikely to cause human illness. Despite the above, the levels of *L. monocytogenes* reached in some treatments in this study may cause infection in some susceptible consumers however, when realistic initial contamination levels (≤10 CFU.g^{-1}) are considered growth of *L. monocytogenes* could be completely inhibited or limited to levels that would not possibly be an unacceptable risk to consumers. Further research could be aimed at isolating and identifying LAB, such as *Lb. sakei*, with low spoilage potential that could act as bio-control bacteria (sometimes called 'protective cultures') on MAP ham to improve the control of *L. monocytogenes*. Such research would require shelf life trials for ham products inoculated with high levels of *Lb. sakei* to determine the effect of those cultures on product quality over time.

Introduction of LAB, that do not spoil the product and have faster growth rates than *L. monocytogenes* at refrigeration temperatures, may provide a natural alternative to chemical treatments to lower the potential for growth of *L. monocytogenes* on ham products. Ultimately a reliable and commercially viable strategy based upon exploitation of the Jameson Effect may become available.

6 Conclusions

L. monocytogenes was not found in the 100 Australian VP/MAP meats surveyed in this study. The results of this survey supported the improved control of *L. monocytogenes* by HACCP and GMP. The survey did, however, detect some other non-pathogenic *Listeria* spp. These mostly related to meats sliced at retail, prior to sale. The presence of these bacteria would still result in costly control procedures which may involve the implementation of a control action plan involving re-testing samples, environmental testing and, in some cases, recalling distributed product. If one *Listeria* spp. is present, *L. monocytogenes* may also be present due to the similar growth characteristics of Listeria *spp.* on processed meats. The presence of any *Listeria* spp. may mean the cleaning and sanitation process was not conducted efficiently. The retail slicer was the main source of Listeria *spp.* contamination in the deli smallgoods, however, only one *Listeria* spp. was found in the 80 VP/MAP products sampled.

The Australian/New Zealand Standard method (AS/NZS 1766.2.16.1:1998) utilised in this study was varied to examine the efficacy of the method. Additional sampling at 24 h of the secondary enrichment broth yielded one positive sample for *Listeria* spp. where the Australian/New Zealand Standard method did not. The effects of bacterial competition may have been underestimated by following only the Australian New Zealand Standard method. The effects of competition in enrichment broths were also explored in this study by spiking meat samples with varying levels of *L. monocytogenes.* In some cases the lower inoculum levels on spiked smallgoods samples were not detected on all selective plates.

The introduction of CHROMagar™Listeria agar decreased the time for *Listeria* spp. detection. This was achieved by eliminating the false positive *L. innocua*, often confused with *L. monocytogenes* using the Australian/New Zealand Standards method. The two bacteria look similar on PALCAM and Oxford agar plates and are not fully identified until the CAMP test towards the end of testing. For example, samples tested using the Australian/New Zealand Standard method took approximately 6-7 days to conclude that the *Listeria* spp. were detected and 7-8 days for identification of a non-

L. monocytogenes spp. This was compared to a shorter detection time of 4-5 days, if *L. monocytogenes* was present, using CHROMagar™Listeria agar. This medium could be used as a third diagnostic medium for detecting *L. monocytogenes* using the Australian/ New Zealand Standard method.

Three LAB were isolated from MAP ham tested in this study, namely *Le. mesenteroides*, *Lc. carnosum* and *Lb. sakei* . Of these three LAB, *Lb. sakei* and *Le. mesenteroides* were studied further by conducting axenic trials as they had the faster growth rates, and were subsequently utilised in bacterial competition studies with *L. monocytogenes*.

L. monocytogenes and other *Listeria* spp. are ubiquitous, thus, control measures are required to inhibit the pathogen in foods. Non-chemical methods were explored in this study by studying the effect of bacterial competition on suppression of the MPD of *L. monocytogenes* via the Jameson Effect. The systematic study of bacterial competition revealed the inhibition of *L. monocytogenes* in co-culture was due to nutrient depletion. Studies in spent broths were undertaken to observe the effect on *L. monocytogenes* MPD when the main substrate for *L. monocytogenes* growth glucose is depleted by other bacteria. The absence of glucose did suppress *L. monocytogenes* growth by 1-1.5 logs however it did not completely inhibit growth of *L. monocytogenes*. From an initial inoculum level of ~10^4 CFU.mL^{-1} an MPD of ~10^8 CFU.mL^{-1} was achieved in spent broths i.e. in the absence of glucose. This may be due to another substrate being utilised or a survival mechanism coming into play. Interestingly, *P. fluorescens* spent broths did not suppress *L. monocytogenes* growth, or MPD. This may have been due to some residual glucose in spent broths or the fact that *P. fluorescens* uses different substrates for growth, thus, competition between the two bacteria did not result. Bacteria that do not compete for the same nutrients as *L. monocytogenes* would not be appropriate to suppress *L. monocytogenes* as a result of the Jameson Effect.

The information gained from studies in the broth system in Chapter 4 was explored further in a food system. This was achieved by inoculating the three LAB and a cocktail of *L. monocytogenes* onto commercially available MAP ham. Addition of the three LAB to endogenous bacteria on MAP ham decreased MPD of *L. monocytogenes* from

an initial level of 10^3 CFU.g^{-1} from 10^4 CFU.g^{-1} (exogenous bacteria alone) to 10^3 CFU.g^{-1} (LAB added) at 4°C and 10^7 CFU.g^{-1} from 10^5 CFU.g^{-1} (exogenous bacteria alone) to 10^3 CFU.g^{-1} (LAB added) at 8°C. This was also seen at 4°C, however, less inhibition was observed due to slower growth of *L. monocytogenes* at that temperature. Therefore, in all cases a decrease of 1 to 2 logs was observed with the addition of LAB. The Jameson Effect was further emphasised when compared to Tamplin (*pers. comm.*) where the growth of *L monocytogenes* on sterile ham (no microorganisms) was as high as 9.66 logs at 5°C. Realistic *L. monocytogenes* levels found in smallgoods are typically <100 CFU.g^{-1}. Thus, suppression of potential *L. monocytogenes* growth might be even greater in naturally contaminated products compared to that observed in the challenge trials reported here.

Microbial competition between LAB and *L. monocytogenes* was examined directly in a food system and indirectly by broth studies. For studies in the food system, interactions were explored by addition of cultured *L. monocytogenes* to ham containing naturally present LAB and by addition of cultured *L. monocytogenes* and LAB that had been previously isolated from ham. The broth system explored basic factors effecting *L. monocytogenes* MPD such as nutrient depletion and pH. The broth system did not, however, consider other variables important in a food system such as meat constituents and chemical metabolites (e.g. level of dissociated lactic acid). While the data from both studies may not be compared directly, the following observations were similar between the broth and food systems. The spent broth trials showed inhibition of *L. monocytogenes'* MPD when grown in spent broth of its own, or in *E. coli*, *Le. mesenteroides* and *Lb. sakei* spent broth. This inhibition was also observed in the ham food system, however, a greater decline in *L. monocytogenes* MPD occurred when *Le. mesenteroides*, *Lb. sakei* and *Le. carnosum* were added to ham with naturally occurring endogenous bacteria. The utilisation of nutrients (e.g. glucose) by competing bacteria appeared to decrease the MPD of *L. monocytogenes* irrespective of the low pH caused by bacteria metabolism. Low pH was observed in the food and broth systems after *L. monocytogenes* was first observed. However, the effect of pH and nutrient depletion on inhibition could only be observed directly in the broth system as pH and nutrient levels were varied.

The effect of LAB and *L. monocytogenes* growth rates and the temperature at which one bacterium will out compete another bacterium was explored using the $T_{equivalent}$ concept. $T_{equivalent}$ is a new concept which requires further research to develop statistically significant results. From this study it was observed that *Lb. sakei* may grow faster than *L. monocytogenes* on MAP ham until the temperature exceeds 10°C. As the temperature of smallgoods in retail storage is reported to be between 2.3 and 10.4°C (Ross et al. 2004), inhibition of *L. monocytogenes* via *Lb. sakei* is likely under these conditions. Future research could involve conducting more observations at 10, 15 and 20°C to provide statistically significant results. Similar $T_{equivalent}$ temperatures (i.e. 10 and 15°C) were estimated for endogenous LAB and added LAB against *L. monocytogenes*. This may suggest the dominant LAB present on the MAP ham when MPD was reached was *Lb. sakei*. *Lb. sakei's* faster growth rate and ability to grow at 4°C may have resulted in it being the main LAB present. This was supported by higher numbers of LAB observed in trials with added LAB compared to exogenous LAB alone, where other micro-organisms appeared to dominate.

Introduction of LAB that grow at low temperatures but do not spoil smallgoods products and have a faster growth rate than *L. monocytogenes* may provide a natural alternative to chemical treatments to lower the potential for *L. monocytogenes* growth on MAP ham products. However, the Jameson Effect may not suppress *L. monocytogenes* when temperatures are higher than 10°C, i.e. if temperature abuse occurs.

The role of lactic acid bacteria in suppression the growth of *L. monocytogenes* in VP/MAP should be considered when estimating the risk posed from *L. monocytogenes* in processed meats.

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Appendices

A1 Strain details, maintenance and recovery

A1.1 Bacterial strains

Inoculation studies

L. monocytogenes (Scott A)
Type strain supplied by the University of Tasmania, GPO Box 252/54, Hobart, TAS, 7001.

L. monocytogenes (L5/22)
A wild type cold smoked salmon isolate (L5/22), courtesy of Dr S. Tienungoon, University of Tasmania.

L. monocytogenes (20425)
L. monocytogenes (20432)
L. monocytogenes (20423)
Wild type isolates from an undisclosed smallgoods factory courtesy of Silliker Microtech Pty. Ltd. The isolates 20425, 20432 and 20423 originated from sites as follows: 20425-swab from the floor outside the smokehouse; 20432-swab from platform handrail and 20423-swab from floor of slicing chiller. All isolates were identified as Listeria *monocytogenes* by Silliker Microtech using gene probes.

Leuconostoc mesenteroides
Leuconostoc carnosum
Lactobacillus sakei
All strains were isolated from two MAP ham products as described in Chapter 3 and were stored at the School of Agricultural Science, University of Tasmania.

Bacteriocin assay

Lactobacillus plantarum (ALC01)
Commercial anti-listerial starter culture Visbyvac DIP Dosis 100-L72722076 obtained from Applied Technical Products P/L, 121 Lewis Rd, Wantirna South,VIC, 3152.

CAMP test

Listeria innocua, ACM 9948
Listeria ivanovii, ACM 3179
Listeria monocytogenes, ACM 98
Rhodococcus equi, ACM 702
Staphylococcus aureus, ATCC 25925
All strains were obtained from the University of Tasmania, GPO Box 252/54, Hobart, TAS, 7001.

A1.2 Cryogenic storage

A1.2.1 Bead cultures

Bacterial cultures were stored at -70°C. Cultures were maintained in duplicate, with one set held for subculture purposes only.

Plastic 3mm beads were cleaned with a tap water and detergent wash followed by a rinse in dilute HCl to neutralise alkalinity. The beads were then washed several times in tap water, followed with a final distilled water rinse and air-drying. Approximately 20-30 beads were placed in 5mL screw-cap glass vials and sterilised by autoclaving at 121°C at 15 psi for 15 minutes. Three vials were reserved for stock cultures and another three vials were used for recovery culture.

Each bacterial strain was grown for 24 h on Nutrient Agar at 37°C. The plate was harvested by pipetting 1mL of sterile Nutrient Broth with 15% glycerol added (NB-Gly) onto the surface of the plate then emulsifying the growth with a wire loop. The bacterial suspension was aseptically pipetted into two vials containing sterile prepared beads. The suspension was aspirated several times to ensure displacement of air in the beads. Excess suspension was removed then vials were placed on their sides and the beads distributed by gentle tapping in order to facilitate easy removal. Vials were placed at -20°C for 24 hours before transfer to -70°C for storage up to 7 years.

A1.2.2 Glycerol suspensions (LAB only)

1. Sterilize eppendorfs and make up nutrient broth.
2. Add 30% glycerol to nutrient broth and sterilize.
3. Use fresh cultures on plates, 24 h on TSB at 25 °C.
4. Add 1-2ml of nutrient broth to plates and using sterilized glass rod (flame with ethanol) smooth liquid around to form a solution of culture and broth.
5. Take up 1ml from plate and place in eppendorfs (label all eppendorfs).
6. Store in -70°C for long term storage.

A1.3 Recovery from cryogenic storage

Cultures on beads were recovered by aseptically removing one bead from the vial while on ice in order to prevent excessive heating of the culture. The bead was rubbed gently over the surface of a Tryptone Soya Agar Broth + 0.6% Yeast Broth (TSB-Ye) plate. The inoculated plate was incubated at 37°C for 24-48 h. The culture was checked visually for purity (colony morphology only) then plated onto an appropriate medium to test for typical reactions.

LAB isolates were resuscitated from –70°C storage as follows. Samples were allowed to rapidly defrost at room temperature (~ 25-38°C). A loopful of cryogenic culture was streaked onto the surface of an MRS plate and incubated at 25°C for 24h. A single colony was inoculated into 10 ml MRS broth and incubated for a further 20 h to provide a stationary phase culture of ~10^9 cfu.mL^{-1}/ml.

A2 Equipment

API computer software program: bioMérieux Inc. 595 Anglum Rd, Hazelwood, MO 63042-2320, USA.

Cricket Graph: A regression-fitting software package. Version 1.3.2. Cricket Software, Philadelphia, USA.

Centrifuge: HeHich, 0034-GBK, Universal 16A (5000RPM), 8x60g.

Glucose meter: Blood glucose Glucose Analyser VSI 2300 STAT Plus-O, YSI Incorporated. 1700/1725 Brannum Lane, Yellow Springs, Ohio, 45387, USA. Courtesy of the Biochemistry Department, University of Tasmania, GPO Box 252/54, Hobart, TAS, 7001.

L tubes: Test tubes with a 90 degree bend with a 40mL capacity.

pH Meter and electrode: Orion pH meter 250A (Orion Research Inc. Boston, MA 02129, USA), and flat tip probe.
The instrument was calibrated on each occasion before use by reference to buffers at pH 4 and pH 7.

Pipettors: 100 µL and 1000 µL (Gilson Medical Electronics, B.P. 45, F95469), 0.01-10.00 mL-electronic digital pipette (Rainin Instrument Co., Inc., 5400 Hollis St, Emeryville, CA 94608-2508.
The dispensed volume of all pipettors was checked by weighing of water at room temperature prior to use. This volume was typically found to be within ±1% of the nominal volume.

Spectrophotometer-digital: Spectronic 20D, Milton Roy Co., USA

Spiral Plater: Autoplate 4000. Spiral Biotech Inc., 7830 Old Georgetown Rd, Bethesda, MD 20814 USA

Stomacher: Colworth 400, A.J. Seward, London, UK.

Stomacher Bags-17.7x30.0cm: Bio-Service Pty. Ltd., P.O. Box 180, Huntingdale, Vic, 3166, Aus.

Thermal Gradient Incubator: Terratec Asia-Pacific Pty., Ltd. Lot 7, Patriarch Dr, Kingston, Tasmania, 7054, Australia.

Thermometer: Fluke® 51K/J (John Fluke Mfg. Co. Inc., 1150Euclid Avenue, Palatine, IL 60067, USA) electronic thermometer with Iron-Constantan thermocouple bead probe.

UltraFit: A non-linear curve fitting package (Biosoft. PO Box 10938, Ferguson MO 63135 USA)

Vortex mixer: Model MT19 (Chiltern Scientific). Variable speed control from 300 to 2200 rpm.

Water Activity Meter: Aqualab CX-2 (Decagon Devices, Inc., PO Box 835, Pullman, Washington 99163, USA).
The instrument was calibrated on each occasion before use by reference to distilled water and to a saturated salt (NaCl, a_w 0.953) standard.

Waterbath: Ratek SWB20D shaking waterbaths, Ratek Instruments Pty. Ltd., Unit 1/3 Wadhurst Drive, Boronia, VIC, Australia, 3155.

A3 Media

A3.1 Media preparation

Basal media was prepared as per manufacturers instructions unless otherwise stated. Addition of supplements was as per manufacturers instructions or as described in this section. Sterilisation was by autoclaving at 121°C at 15 psi for 15 minutes (unless otherwise specified) or in the case of non-sterile, heat sensitive supplements by filter sterilisation. Where necessary pH was modified by the addition of 0.1M NaOH or 0.1M HCl unless otherwise stated. pH was measured post-autoclaving and adjusted aseptically by the addition of sterile HCl or NaOH if required. For NaCl modified media, a_w was determined from triplicate readings. All water used in the preparation of media was prepared by glass distillation of tap water.

Quality control was exercised on each batch of media prepared by:

- measurement of media pH after sterilisation
- incubation of an uninoculated plate at 25°C for 40 to 48 h as a sterility check
- growth of target organism at optimal temperature

Media failing these criteria were discarded.

Media were stored at 4°C for up to 4 weeks. Media containing active selective ingredients were stored in the dark at 4°C for up to 2 weeks.

A3.2 Media used

Brain Heart Infusion Agar (BHI)

Brain Heart Infusion Agar (BHI)(CM375, Oxoid)	47g
Distilled water	1000 mL

Blood Agar plates:

Columbia Agar base (Oxoid, CM331)	36.5-45.5g
Defibrinated sheep blood (Oxoid)	50 mL
Distilled water	1000 mL

CFC (Pseudomonas) agar

CFC (CM559, Oxoid)	24.2g
Distilled water	500 mL
Glycerol	5 mL
C-F-C selective supplement (SR103E, Oxoid)	

Eosin Methylene Blue Agar (EMB)

Eosin Methylene Blue Agar-Levine (Oxoid CM69)	37.5 g
Distilled Water	1000 mL

After autoclaving, the agar medium was cooled to 60 °C and shaken to oxidise the methylene blue and redistribute the precipitate. The agar media was then further cooled to 50°C prior to pouring plates.

Fraser broth (FB)

Fraser Broth (Oxoid, CM877B)	57.4g
Fraser Selective Supplement (SR156E)	1 vial
Distilled water	1000 mL

Half Fraser Broth (1/2FB)

Fraser Broth (Oxoid, CM877B)	13.35g
Fraser supplement – half (Oxoid, SR166E)	1 vial
Distilled water	225 mL

De Man, Rogosa Sharpe (MRS) broth

De Man, Rogosa Sharpe (MRS) (CM359, Oxoid)	52 g
Distilled Water	1000 mL

De Man, Rogosa Sharpe (MRS) agar

De Man, Rogosa Sharpe (MRS) (CM359, Oxoid)	52 g
Distilled Water	1000 mL
Agar	15g

Nutrient Agar (NA)

Nutrient Broth (Oxoid CM 1)	13 g
Agar	15g
Distilled Water	1000 mL

Nutrient Broth with 15% v/v glycerol (NB-Gly)

Nutrient Broth (Oxoid CM 1)	13 g
Glycerol (BDH-Anala R, Prod 10118)	15 mL
Distilled Water	1000 mL

15mL of glycerol was measured into a volumetric flask then dispensed into a vessel containing the NB powder, but without the distilled water. Due to the viscous nature of the glycerol, small volumes of the distilled water were added to the volumetric flask to "wash out" the glycerol into the vessel containing the NB.

Nutrient Broth (NB)

Nutrient Broth (Oxoid CM 1)	13.0 g
Distilled Water	1000 mL

Plate Count Agar (PCA)

Plate Count Agar Standard-APHA (Oxoid CM 463)	23.5 g
Distilled Water	1000 mL

PALCAM agar

PALCAM selective supplement (Oxoid, SR150E)	34.5g
PALCAM agar base (Oxoid, CM877B)	1 vial
Distilled Water	500 mL

Oxford Listeria agar

Listeria selective supplement base (Oxoid, CM856)	27.75g
Listeria selective supplement (Oxoid, SR156E)	1vial
Distilled Water	500 mL

Tryptone Soya Broth + 0.6% Yeast Extract (TSB-Ye) broth

Tryptone Soya Broth (Oxoid CM 129)	30.0 g
Yeast Extract (Oxoid L21)	6.0 g
Distilled Water	1000 mL

Tryptone Soya Broth + 0.6% Yeast Extract (TSB-Ye) agar

Tryptone Soya Broth (Oxoid CM 129)	30.0 g
Yeast Extract (Oxoid L21)	6.0 g
Distilled Water	1000 mL
Agar	15g

Carbohydrate test
The number of isolates required for testing was determined and appropriate numbers of CHO tubes prepared. For 100 isolates to be tested with both sugars, 600 mL of medium was made to dispense in tubes of 3 mL aliquots. Thus ingredients required were:

6g Protease peptone
0.6g meat extract
3g NaCl
0.006g Bromocresol purple
600 mL Water

Rhamnose and xylose was added to each tube (Aliquots of 300μL).

A3.3 CHROMagar™Listeria.

CHROMagar™Listeria (LM85-011108) (CHROMagar Microbiology, 4, place du 18 juin 1940 75006 Paris France. Fax (33- 45 48 06 00) procedure manual.
Composition in $g.L^{-1}$: Agar, 15; Peptones and meat extracts, 23; NaCl, 5; Chromogenic mix, 17.5;pH 7.0.
Preparation for 250 mL vials melting of base:

- 250 mL purified water (distilled or deionized) in a vial.
- Disperse slowly "CHROMagar Listeria base ref LM850(B) powder in the water by rotating until swelling of the agar.
- Autoclave at 121°C, 15mins. Cool in a water bath to 48 °C.

A4 Other tests

A4.1 Gram stain

A suspension of bacteria was smeared onto a glass slide, allowed to dry and heat fixed. It was then flooded with crystal violet 1g/100 mL distilled water) for one minute, washed with water, flooded with iodine (1g/300 mL distilled water) for one minute, washed with acetone or alcohol to decolourise (100 μL), washed and counterstained with safranin (1g/100mL) for one minute, washed and allowed to dry.

A4.2 Motility

Motility test for *L. monocytogenes* was tested as follows. The isolate was grown in Nutrient Broth for 6 h at 37°C. A small drop was placed on a microscope slide and observed using phase contrast.

A4.3 API test strip carbohydrate profile.

API 50 CH strips (50 410), bioMérieux Inc. Each well consisted of a carbohydrate below. Wells were scored between 0-5 where 4-5 was yellow and 3 was green. Anything above 3 was considered a positive result. The code was entered into computer using API identification software, bioMérieux.

Table A.1 Carbohydrate profile for API 50 CH strips.

Carbohydrate	
1. Control	26. Esculine
2. Glycerol	27. Salicine
3. Erthritol	28. Cellobiose
4. D-Arbinose	29. Maltose
5. L -Arabinose	30. Lactose
6. Ribose	31. Melibiose
7. D -xylose	32. Saccharose
8. L-xylose	33. Tehalose
9. Adonitol	34. Inuline
10. β Methyl-xyloside	35. Melezitose
11. Galactose	36. D-Raffinose
12. D-Glucose	37. Amidon
13. D-Frucotse	38. Glycogene
14. D-Mannose	39. Xylitol
15. L-Sorbose	40. β Gentiobiose
16. Rhamnose	41. D-Turanose
17. Dulcitol	42. D-Lyxose
18. Inositol	43. D-Tagatose
19. Mannitol	44. D-Fucose
20. Sorbitol	45. L- Fucose
21. α Methyl-D-mannoside	46. D-Arabitol
22. α Methyl0d-glucosamine	47. Gluconate
23. N Acetyl glucosamine	48. 2 ceto-gluconate
24. Amygdaline	49. 5 ceto-gluconate
25. Arbutine	

A5 Raw experimental data

A5.1 Chapter 1: Smallgoods survey

Table A.2 Smallgoods sample description and characteristics for mid summer (13/12/01). ND=Not detected

Number	Product Name	Company	Days Left at 4°C to 8°C	pH (water)	Water Activity	Listeria species
	Deli meats					
1	Mortadella plain	Tabaldi	NA	6.45	0.958	ND
2	Pancetta mild	Don	NA	5.7	0.939	*L. welshimeri or L. denitrificans*
3	Ox tongue	Don	NA	6.36	0.980	ND
4	Mordella plain half	Don	NA	6.27	0.961	*L. welshimeri* or *L. denitrificans*
5	Tibaldi proscuitto parma	Don	NA	5.99	0.740	ND
	VP/MAP RTE meats					
6	Shaved leg ham	KR darling downs	18 days	6.27	0.958	ND
7	Shaved leg ham	KR darling downs	18 days	6.23	0.956	ND
8	Shaved leg ham	KR darling downs	18 days	6.26	0.957	ND
9	Shaved leg ham	KR darling downs	18 days	6.26	0.957	ND
10	Shaved leg ham	KR darling downs	18 days	6.3	0.955	ND
11	Prosciutto	San marino prosciutto	91 days	6.18	0.872	*L. welshimeri or L. denitrificans*
12	Prosciutto	San marino prosciutto	91 days	6.22	0.873	ND
13	Prosciutto	San marino prosciutto	91 days	6.19	0.884	ND
14	Prosciutto	San marino prosciutto	91 days	6.2	0.884	ND
15	Prosciutto	San marino prosciutto	91 days	6.46	0.891	ND
16	Pate cracked pepper	Jean pierre pate	14 days	6.36	0.974	ND
17	Pate cracked pepper	Jean pierre pate	14 days	6.32	0.988	ND
18	Pate cracked pepper	Jean pierre pate	14 days	6.32	0.985	ND
19	Pate cracked pepper	Jean pierre pate	14 days	6.31	0.986	ND
20	Pate cracked pepper	Jean pierre pate	14 days	6.31	0.982	ND
21	Sliced honey ham	Echovalley	17 days	5.78	0.972	ND
22	Sliced honey ham	Echovalley	17 days	5.8	0.972	ND
23	Sliced honey ham	Echovalley	17 days	5.83	0.972	ND
24	Sliced honey ham	Echovalley	17 days	5.94	0.972	ND
25	Sliced honey ham	Echovalley	17 days	5.9	0.973	ND

Table A.3 Smallgoods sample description and characteristics for midsummer at Coles Supermarket (17/12/01). ND=Not detected

Number	Product Name	Company	Days Left at 4°C to 8°C	pH (water)	Water Activity	Listeria species
	Deli meats					
21	Ham Honey	NR	17/12/01 (packed)	6.51	0.977	*L. innocua*
22	Pork roast	NR	17/12/01 (packed)	6.29	0.968	*L. innocua*
23	Roast Turkey	NR	17/12/01 (packed)	6.65	0.974	*L. innocua*
24	Silver side	Blue Ribbon	17/12/01 (packed)	6.27	0.98	*L. innocua*
25	Roast Beef	Melbourne	17/12/01 (packed)	6.24	0.966	*L. innocua*
	VP/MAP RTE meats					
1	Farmland Breakfast Bacon	Farmland	25 days	6.28	0.972	ND
2	Farmland Breakfast Bacon	Farmland	25 days	6.12	0.971	ND
3	Farmland Breakfast Bacon	Farmland	25 days	6.43	0.97	ND
4	Farmland Breakfast Bacon	Farmland	25 days	5.77	0.972	ND
5	Farmland Breakfast Bacon	Farmland	25 days	5.98	0.968	ND
6	Pate	South cape	26 days	6.28	0.987	ND
7	Pate	South cape	26 days	6.3	0.987	ND
8	Pate	South cape	26 days	6.26	0.986	ND
9	Pate	South cape	26 days	6.26	0.987	ND
10	Pate	South cape	26 days	6.28	0.986	ND
11	Shaved baked English leg ham	Makers choice	19 days	6.36	0.968	ND
12	Shaved baked English leg ham	Makers choice	19 days	6.3	0.969	ND
13	Shaved baked English leg ham	Makers choice	19 days	6.37	0.968	ND
14	Shaved baked English leg ham	Makers choice	19 days	6.46	0.969	ND
15	Shaved baked English leg ham	Makers choice	19 days	6.45	0.966	ND
16	Don unsmoked Ham leg	Don	14 days	6.47	0.968	ND
17	Don unsmoked Ham leg	Don	14 days	6.42	0.97	ND
18	Don unsmoked Ham leg	Don	14 days	6.39	0.969	ND
19	Don unsmoked Ham leg	Don	14 days	6.35	0.971	ND
20	Don unsmoked Ham leg	Don	14 days	6.37	0.968	ND

Table A.4 Smallgoods sample description and characteristics late summer (21/2/02). ND=Not detected

Sample	Product Name	Company Name	Days Left at 4°C to 8°C	Water Activity	pH (water)	Listeria present
	Deli Meat					
1	Mt Nelson ham	NR		0.98	6.37	ND
2	Mt Nelson ham on leg	NR		0.97	6.58	ND
3	Mt Nelson roast turkey	NR		0.98	6.49	ND
4	Mt Nelson chicken	NR		0.97	6.46	ND
5						
	VP/MAP RTE meats					
6	Leg ham, champagne	Primo	25 days	0.97	6.52	Contamination control
7	Leg ham, champagne	Primo	25 days	0.97	6.46	Contamination control
8	Leg ham, champagne	Primo	25 days	0.97	6.43	ND
9	Leg ham, champagne	Primo	25 days	0.97	6.40	ND
10	Leg ham, champagne	Primo	25 days	0.97	6.39	ND
11	Shaved Italian style mortadella	Makers Choice	29 days	0.98	6.53	ND
12	Shaved Italian style mortadella	Makers Choice	29 days	0.98	6.50	ND
13	Shaved Italian style mortadella	Makers Choice	29 days	0.98	6.53	ND
14	Shaved Italian style mortadella	Makers Choice	29 days	0.98	6.50	Contamination control
15	Shaved Italian style mortadella	Makers Choice	29 days	0.98	6.42	Contamination control
16	Sliced ham	Echo Valley	25 days	0.98	6.25	ND
17	Sliced ham	Echo Valley	25 days	0.98	6.16	ND
18	Sliced ham	Echo Valley	25 days	0.98	7.02	ND
19	Sliced ham	Echo Valley	25 days	0.98	6.16	ND
20	Sliced ham	Echo Valley	25 days	0.98	6.10	ND
21	Lean and tasty, 975 fat free ham	Hans	32 days	0.97	6.47	ND
22	Lean and tasty, 975 fat free ham	Hans	32 days	0.97	6.44	ND
23	Lean and tasty, 975 fat free ham	Hans	32 days	0.97	6.46	ND
24	Lean and tasty, 975 fat free ham	Hans	32 days	0.97	6.44	ND
25	Lean and tasty, 975 fat free ham	Hans	32 days	0.97	6.50	ND

Table A.5 Smallgoods sample description and characteristics during mid-summer (10/12/02). ND=Not detected

Sample	Product Name	Company Name	Days Left at 4°C to 8°C	Water Activity	pH (water)	Listeria detected
	Deli meat					
1	Chicken		84 days	0.982	6.32	ND
2	Ham		84 days	0.972	6.46	ND
3	Silver side		84 days	0.975	6.23	ND
4	Turkey		84 days	0.979	6.29	ND
5	Honey ham		84 days	0.978	5.99	ND
	VP/MAP RTE meats					
6	Pastrami	Don	88 days	0.970	5.46	ND
7	Pastrami	Don	88 days	0.972	5.26	ND
8	Pastrami	Don	88 days	0.970	5.30	Spiked control
9	Pastrami	Don	88 days	0.970	5.39	ND
10	Pastrami	Don	88 days	0.972	5.24	ND
11	Champagne ham	Freshpak	105 days	0.970	5.64	ND
12	Champagne ham	Freshpak	105 days	0.973	5.61	ND
13	Champagne ham	Freshpak	105 days	0.971	5.56	ND
14	Champagne ham	Freshpak	105 days	0.971	5.57	ND
15	Champagne ham	Freshpak	105 days	0.973	5.57	ND
16	Sandwich slice mixed meats	Blue Ribbon	94 days	0.972	5.70	ND
17	Sandwich slice mixed meats	Blue Ribbon	94 days	0.978	5.62	ND
18	Sandwich slice mixed meats	Blue Ribbon	94 days	0.978	5.73	ND
19	Sandwich slice mixed meats	Blue Ribbon	94 days	0.981	5.97	ND
20	Sandwich slice mixed meats	Blue Ribbon	94 days	0.974	5.57	ND
21	Kabanos, meat including pork	Don	115 days	0.967	5.73	ND
22	Kabanos	Don	115 days	0.973	5.72	ND
23	Kabanos	Don	115 days	0.972	5.65	ND
24	Kabanos	Don	115 days	0.966	5.69	ND
25	Kabanos	Don	115 days	0.967	5.46	ND

A6 LAB calculations of generation time and Lag times (Chapter 3)

Table A.6 Generation time (GT) and sqrt(1/GT) of *Leuconostoc mesenteroides.*

Temperature °C	Generation time (mins)	sqrt(1/GT)	Lag time (mins)
5	15050	0.008	3928
7.4	6020	0.013	-731
9.7	3010	0.018	862
12.7	3010	0.018	-812
15.1	1003	0.032	66
17.8	1003	0.032	-208
20.5	501	0.045	58
22.6	376	0.052	65
25	334	0.055	20
27.3	215	0.068	56
29.6	200	0.071	77
31.8	167	0.077	21

Table A.7 Generation time (GT) and sqrt(1/GT) of *Leuconostoc carnosum.*

Temperature °C	Generation time (mins)	sqrt(1/GT)	Lag time (mins)
6.10	7525	0.012	1408
8.50	4300	0.015	922
11.50	3101	0.018	197
13.50	1505	0.026	139
16.40	1003	0.032	152
17.70	1003	0.032	120
21.30	602	0.041	103
23.40	602	0.041	143
25.10	501	0.045	140
27.90	602	0.041	83
30.40	752	0.036	72
32.20	501	0.045	778

Table A.8 Generation time (GT) and sqrt (1/mins) for *Lactobacillus sakei.*

Temperature °C	Generation time (mins)	sqrt(1/GT)	Lag time (mins)
4.7	3101	0.018	96
7.2	5016	0.014	-127
10.8	1505	0.026	622
13.4	1003	0.032	435
16	752	0.036	437
19	376	0.052	262
20.4	430	0.048	129
22.5	334	0.055	79
27.6	273	0.061	29
29.8	273	0.061	24
32.1	231	0.066	24

Table A.9 A summary of GT (mins) values at 4°C and 8°C.

LAB species	gradient	Temp (degrees)	y-intercept	sqrt(1/GT)	Average of duplicate	GT (mins)
Leuconostoc mesenteroides	0.0047	4.00	-0.0154	0.0034		
	0.0048	4.00	-0.0159	0.0033	0.00335	89106
	0.0047	8.00	-0.0154	0.0222		
	0.0048	8.00	-0.0159	0.0225	0.02235	2002
Leuconostoc carnosum	0.0030	4.00	-0.0060	0.006		
	0.0030	4.00	-0.0057	0.0063	0.00615	26439
	0.0030	8.00	-0.0060	0.018		
	0.0030	8.00	-0.0057	0.0183	0.01815	3036
Lactobacillus sakei	0.0026	4.00	0.0119	0.0223		
	0.0027	4.00	0.0107	0.0215	0.0219	2085
	0.0026	8.00	0.0119	0.0327		
	0.0027	8.00	0.0107	0.0323	0.0325	947

Table A.10 The generation times, growth rate(1/GT) and Sqrt (1/GT) for *L. monocytogenes* (Scott A) in TSB-Ye, pH 5.5, a_w 0.970 (Tienungoon, 1998).

T °C	Sqrt (rate)	Rate(1/GT)	GT (mins)
4	0.085	0.007	137.815
5	0.112	0.013	79.033
6	0.140	0.020	51.176
7	0.167	0.028	35.818
8	0.194	0.038	26.463
9	0.222	0.049	20.347
10	0.249	0.062	16.129
11	0.276	0.076	13.099
12	0.304	0.092	10.849
13	0.331	0.109	9.133
14	0.358	0.128	7.794
15	0.386	0.149	6.729
16	0.413	0.170	5.868
17	0.440	0.194	5.163
18	0.467	0.218	4.577
19	0.495	0.245	4.086
20	0.522	0.272	3.670
21	0.549	0.302	3.314
22	0.577	0.332	3.008
23	0.604	0.365	2.742
24	0.631	0.398	2.510
25	0.658	0.434	2.307
26	0.686	0.470	2.127
27	0.713	0.508	1.968
28	0.740	0.547	1.827
29	0.767	0.588	1.701
30	0.793	0.629	1.589

Table A.11 Volume required to achieve LAB starting numbers.

Species	**Initial volume**	**Average %T Scale 50%**
Leuconostoc mesenteroides	1 ml	43.5
Leuconostoc carnosum	1 ml	45
Lactobacillus curvatus	1.5 ml	32.8

Table A.12 Actual temperatures of un-inoculated MRS in L-tubes placed in temperature gradient incubator after 24 h equilibrating time.

Tube number	*Leuconostoc mesenteroides*	*Leuconostoc carnosum*	*Lactobacillus sakei*
1	5 °C	6.1 °C	4.7 °C
2	7.4 °C	8.5 °C	7.2 °C
3	9.7 °C	11.5 °C	10.8 °C
4	12.7 °C	13.5 °C	13.4 °C
5	15.1 °C	16.4 °C	16 °C
6	17.8 °C	17.7 °C	19 °C
7	20.5 °C	21.3 °C	20.4 °C
8	22.6 °C	23.4 °C	22.5 °C
9	25 °C	25.1 °C	27.6 °C
10	27.3 °C	27.9 °C	29.8 °C
11	29.6 °C	30.4 °C	32.1 °C
12	31.8 °C	32.2 °C	

A7 Spent broth trails (Chapter 4)

Table A.13 Log values of *Listeria monocytogenes* grown in *Listeria monocytogenes* spent media broth. Spent broth grown for 24 h.

TIME (h)	PH7/TSB-Ye	TSB-Ye	PH7	Spent
0.25	5.13	5.31		5.34
4	5.11	5.84	5.59	5.61
9	5.65	6.76	5.74	5.71
21	8.18	9.26	7.56	7.15
26	9.18	9.51	7.53	7.29
31	9.23	9.54	7.59	
44	9.68	9.66	7.32	7.35
55		9.60	8.11	

Table A.14 Log values of *Listeria monocytogenes* grown in *E. coli* spent media broth. Spent broth grown for 24h.

TIME (h)	Ph7/TSB-Ye	TSB-Ye	PH7	Spent
0.25	5.72	5.88	5.53	5.54
4	5.72	5.88	5.53	5.54
9	6.50			5.72
21	9.00	9.07	7.86	7.77
26	9.46	9.08		8.11
31	10.08	8.40		
44	9.40	8.86		
55				

Table A.15 Log values of *Listeria monocytogenes* grown in *Pseudomonas fluorescens* spent broth. Spent broth grown for 24h.

TIME (h)	PH7/TSB-Ye	TSB-Ye	PH7	Spent
0.25	5.80	5.57	5.90	6.20
4	5.80	5.57	5.90	6.03
9	7.26	7.23	7.10	
21	9.54	9.48	8.83	8.88
26	9.62	9.38	9.77	8.73
31	9.63	9.66		
44	9.49	9.26	8.15	9.23
55				

Table A.16 The pH values for *L. monocytogenes* grown in *L. monocytogenes* spent broth. Spent broth grown for 24 h.

Time (h)	PH7/TSB-Ye	TSB-Ye	PH7	Spent
0	7.2	6.8	7.3	6.4
0.25	7.2	6.8	7.6	6.3
4	7.1	6.8	7.9	6.3
9	7.2	6.8	8.0	6.3
21	7.1	6.4	8.1	6.3
26	6.6	6.0	8.2	6.3
31	6.3	6.0	8.2	6.3
44	6.5	6.8	8.2	6.3
55		6.9	8.1	6.2

Table A.17 The pH values for *L. monocytogenes* grown in *E. coli* spent broth. Spent broth grown for 24hrs.

Time (h)	pH7/TSB-Ye	TSB-Ye	pH7	spent
0	7.22	7.35	7.22	7.22
0.25	7.32	7.35	7.25	7.27
4	7.36	7.34	7.26	7.28
9	7.34	7.35	7.3	7.29
21	6.34	6.35	6.98	6.94
26	6.27	6.27	6.93	6.93
31	6.31	6.26	6.95	6.94
44	6.28	6.26	6.9	6.92
55				

Table A.18 The pH values for *L. monocytogenes* grown in *P. fluorescens* spent broth. Spent broth grown for 24 h.

Time (hours)	pH7/TSB-Ye	TSB-Ye	pH7	spent
0	7.21	6.89	7.22	6.65
0.25	7.23	6.89	7.35	6.57
4	7.27	6.92	7.41	6.59
9	7.31	6.94	7.44	6.61
21	6.71	6.73	7.51	6.86
26	6.99	7.29	7.72	7.68
31	7.27	7.56	7.95	7.77
44	7.36	7.73	8.33	7.85
55				

Table A.19 The growth of *L. monocytogenes* in *L. monocytogenes* spent broth. Spent broth grown until 18 h (partial spent broth) at 25 °C.

TIME (h)	TSB-Ye/pH7	TSB-Ye/spent pH	pH7	Spent
0	4.46	4.51	-	4.54
2	4.68	4.79	4.76	4.52
4	5.43	5.30	5.29	-
6	5.52	5.63	5.56	4.85
8	6.32	6.15	6.38	5.04
10	6.79	6.64	6.53	5.20
15	8.48	8.38	-	5.64
20	8.55	8.59	-	5.94
25	8.45	8.68	8.28	6.18
28.5	8.46	8.79	-	6.36
32.5	8.70	8.69	-	6.32
40.5	-	-	-	6.40
47.5	8.66	8.40	-	6.71
69.5	8.56	8.53	7.52	7.45

Table A.20 The growth of *L. monocytogenes* in *P. fluorescens* spent broth. Spent broth grown until 18 h at 25 °C.

Time (h)	TSB-Ye/pH7	TSB-Ye/spent pH	PH7	Spent
0	-			
2	-	4.23	3.90	3.90
4	4.71	4.67	4.86	4.04
6	5.31	5.23	5.26	
8	6.04	5.92	6.03	
10	6.41	6.29		
15	7.79	7.71		
20	8.30	8.40	8.64	
25	8.52	8.41		
28.5	8.89	8.68	8.83	
32.5	8.98	8.86	8.86	
40.5	8.89	8.49	8.90	5.95
47.5				
69.5	9.29	9.04	8.95	7.00

Table A.21 The %T, glucose and lactate levels when *P. fluorescens* is grown in TSB-Ye at 25 °C.

Rep 1				Rep 2			
%T	pH	glucose (M)	Lactate (M)	%T	pH	glucose (M)	Lactate (M)
94	7.14	10.6	0.237	92	7.14	10.2	0.21
92	7.19	11.5	0.229	90	7.19	10.5	0.299
92	7.19	11.4	0.197	90	7.18	10.9	0.265
90	7.16	10.3	0.266	89	7.18	10	0.262
72	7.07	7.11	0.295	65	7.11	7.26	0.287
58	7.05	8.45	0.066	52	7.09	6.33	0.174
50	7.07	6.45	0.361	43	7.1	6.31	0.306
35	7.07	6.01	0.237	28	7.03	2.96	0.247
24	7	3.88	0.194	18	6.97	1.44	0.199
10	6.9	0.697	0.06	8	6.92	0.256	0.093
8	6.85	0.493	0.102	7	6.94	0.109	0.094
5	6.91	0.143	0.095	4	7.03	0.133	0.116
3.5	6.97	0.102	0.05	3	7.04	0.105	0.083
2	7.02	0.048	0.115	2	7.06	0.114	0.099
0	7.52	0.14	0.06	1	7.38	0.104	0.041
0	8.57	0.144	0.061	1	8.43	0.246	0.08

Table A.22 The Log data for *P. fluorescens* grown in TSB-Ye broth over time at 25 °C.

	Log ABS		pH		Log glucose (M)		Log Lactate (M)	
Abs time	rep 1	rep 2	rep 1	rep 2	rep 1	rep 2	rep 1	rep 2
0	-1.57	-1.44	7.14	7.14	1.025	1.009	-0.625	-0.678
60	-1.44	-1.34	7.19	7.19	1.061	1.021	-0.640	-0.524
180	-1.44	-1.34	7.19	7.18	1.057	1.037	-0.706	-0.577
270	-1.34	-1.30	7.16	7.18	1.013	1.000	-0.575	-0.582
390	-0.85	-0.73	7.07	7.11	0.852	0.861	-0.530	-0.542
420	-0.63	-0.55	7.05	7.09	0.927	0.801	-1.180	-0.759
450	-0.52	-0.44	7.07	7.1	0.810	0.800	-0.442	-0.514
480	-0.34	-0.26	7.07	7.03	0.779	0.471	-0.625	-0.607
510	-0.21	-0.13	7	6.97	0.589	0.158	-0.712	-0.701
570	0.00	0.04	6.9	6.92	-0.157	-0.592	-1.222	-1.032
600	0.04	0.06	6.85	6.94	-0.307	-0.963	-0.991	-1.027
630	0.11	0.15	6.91	7.03	-0.845	-0.876	-1.022	-0.936
690	0.16	0.18	6.97	7.04	-0.991	-0.979	-1.301	-1.081
750	0.23	0.23	7.02	7.06	-1.319	-0.943	-0.939	-1.004
1410	0.30	0.30	7.52	7.38	-0.854	-0.983	-1.222	-1.387
2460	0.30	0.30	8.57	8.43	-0.842	-0.609	-1.215	-1.097

Table A.23 pH values of *L. monocytogenes* grown in *L. monocytogenes* partially spent broth (18 h) at 25°C.

Time (h)	pH7/TSB-Ye added	TSB-Ye/pH unadjusted	pH7	spent
0	7.15	6.49	7.05	5.41
2	7.2	6.55	7.05	5.46
4	7.2	6.56	7.07	5.47
6	7.25	6.58	7.09	5.49
8	7.2	6.62	7.07	5.49
10	7.19	6.57	7.06	5.48
15	6.88	6.45	7.26	5.49
20	6.87	6.08	7.69	5.47
25	7.18	6.6	7.73	5.52
28.5	7.21	7.12	7.98	5.49
32.5	7.3	7.42	8.01	5.5
40.5	7.44	7.63	7.81	5.49
47.5	8.28	7.68	8.39	5.49
69.5	7.69	8.05	8.22	5.71

Table A.24 pH values of *L. monocytogenes* grown in *E. coli* partially spent broth (18 h) at 25°C.

Time (h)	pH7/TSB-Ye added	TSB-Ye/pH unadjusted	pH7	Spent
0	7.03	6.43	7.08	5.47
2	7.09	6.51	7.09	5.47
4	7.1	6.51	7.11	5.48
6	7.12	6.53	7.14	5.5
8	7.1	6.51	7.16	5.51
10	7.1	6.49	7.15	5.49
15	7.18	6.69	7.66	5.5
20	7.75	7.57	8.48	5.5
25	7.74	7.52	8.1	5.51
28.5	7.98	7.77	8.46	5.56
32.5	8.02	7.59	8.44	5.53
40.5	8.2	7.69	8.3	5.53
47.5	7.75	8.28	8.48	5.5
69.5	8.79	7.69	8.63	5.49

Table A.25 *L. monocytogenes* in *L. monocytogenes* spent broth, repeat experiment partially spent broth (18 h) at 25°C.

TIME (h)	TSB-Ye/spent pH	PH7	Spent
0	3.52	4.54	4.68
2.5	4.67		
5	4.86		
7	4.74		
11	5.60	6.23	5.65
13.5	5.38		
21.5	5.90	6.89	5.90
28.5	5.95		
38.5	6.69	6.96	6.66
62	7.52	7.09	6.42
67.5	7.96	7.15	6.89
90.5	7.98		
117	8.03		6.79
140	7.87	7.03	6.95

Table A.26 pH values for repeat *L. monocytogenes* in *L. monocytogenes* partially spent broth (18 h) at 25°C.

Time (h)	TSB-Ye/spent pH	pH7	spent
0	5.54	7.11	5.84
2.5	5.58		
5	5.55		
7	5.56		
11	5.54	7.1	5.85
13.5	5.54		
21.5	5.54	7.11	5.87
28.5	5.5		
38.5	5.5	7.06	5.84
62	5.55	7.09	5.84
67.5	5.15	7.04	5.83
90.5	5.09	7.01	5.83
117	5.08	7.01	5.83
140	5.15	7	5.83

Table A.27 Log values of *Listeria monocytogenes* grown in *Listeria monocytogenes* spent media broth (partial) at 25°C.

Time (h)	Spent, 10^4 CFU.mL^{-1}	Media and pH, 10^4 CFU.mL^{-1}	Media and spent pH, 10^4 CFU.mL^{-1}	PH 7, 10^4 CFU.mL^{-1}	PH spent and nutrient 10^2 CFU.mL^{-1}	Spent, 10^2 CFU.mL^{-1}
0	5.38	5.54	5.30	5.40	3.00	3.70
2	5.52	5.63	5.26	5.91	3.70	3.70
4	6.71	6.58	5.70	6.41	3.60	
6	6.89	6.91	6.20	7.00		
8	7.18	7.48	6.36	7.48		
10	7.32	7.96		7.81		
12	7.64	8.41				
14	8.05	8.95	7.34	8.26		
16	7.76	9.54	7.61	8.23	5.15	
18	7.93	9.92	8.16	8.28		4.72
20	7.93	9.88	8.43	8.52		4.86
22	7.97	9.98	8.66	8.27	6.04	5.00
24	7.81	9.75	8.82	8.18	6.28	5.04
29.5	7.72	9.58	9.09	8.20	6.58	5.51
35		9.86	9.39	8.29	7.20	6.11
40	8.08	9.96	9.62	8.69	7.20	6.20
46.5	7.85	9.98	9.49	8.26	9.16	7.13
50			9.85		9.57	7.70
56	7.99	9.83	9.16	8.53	9.34	7.77
64	8.08	9.85	8.70	8.30	9.56	7.84
90	8.00		9.75	8.24	9.60	8.14
116			9.41		9.57	8.38

Table A.28 The pH values of *Listeria monocytogenes* grown in *Listeria monocytogenes* spent broth (partial) at 25°C.

TIME (h)	Spent, 10^4 CFU.mL^{-1}	Media and pH, 10^4 CFU.mL^{-1}	Media and spent pH, 10^4 CFU.mL^{-1}	PH 7, 10^4 CFU.mL^{-1}	PH spent and nutrient 10^2 CFU.mL^{-1}	Spent, 10^2 CFU.mL^{-1}
0	5.40	7.19	5.73	7.49	5.69	5.45
2						
4	5.47	7.37	5.78	7.83	5.72	5.51
6						
8	5.47	7.35	5.80	7.80	5.76	5.48
10						
12	5.48	7.27		7.66	5.77	5.54
14						
16	5.47	6.84	5.78	7.77	5.73	5.54
18						
20	5.51	6.64	5.83	7.96	5.82	5.58
22						
24	5.49	6.63		7.70	5.78	5.53
29.5						
35	5.58	5.76			5.77	5.56
40						
46.5						
50						
56						
64	5.54	6.55	5.40	7.68	5.31	5.55
90						
116			5.38		5.25	6.94

A8 Ham trial (Chapter 5)

Table A.29 Inoculum levels of *L. monocytogenes* and LAB added to ham

Species	Initial count (CFU.mL^{-1})	Inoculum count (CFU.mL^{-1})
Listeria cocktail	5.1 x 10^8, 3.5 x 10^8	
Listeria cocktail high	-	5.9 x 10^5
Listeria cocktail low	-	5.4 x 10^3
Lactic acid bacteria cocktail	1.6 x 10^8 (MRS)	
	1.8 x 10^8 (PCA)	
Leuconostoc mesenteroides	1.09 x 10^9	
Leuconostoc carnosum	6.1 x 10^7	
Lactobacillus sakei	2.0 x 10^8	

Table A.30 The water activity values for ham products at 8°C and 4°C.

8°C	13 days	47 days	4°C	74 days
C	0.967	0.970	C	0.975
HLC	0.968	0.967	HLC	0.968
LLC	0.967	0.972	LLC	0.971
HLLB	0.968	0.966	HLLB	0.965
LLLB	0.967	0.969	LLLB	0.969
Average	0.9674	0.9688		0.9696

C-control, HLC-high *L. monocytogenes* on control, LLC- low *L. monocytogenes* on control, HLLB-high *L. monocytogenes* on ham with added LAB and LLLB-low *L. monocytogenes* on ham with added LAB.

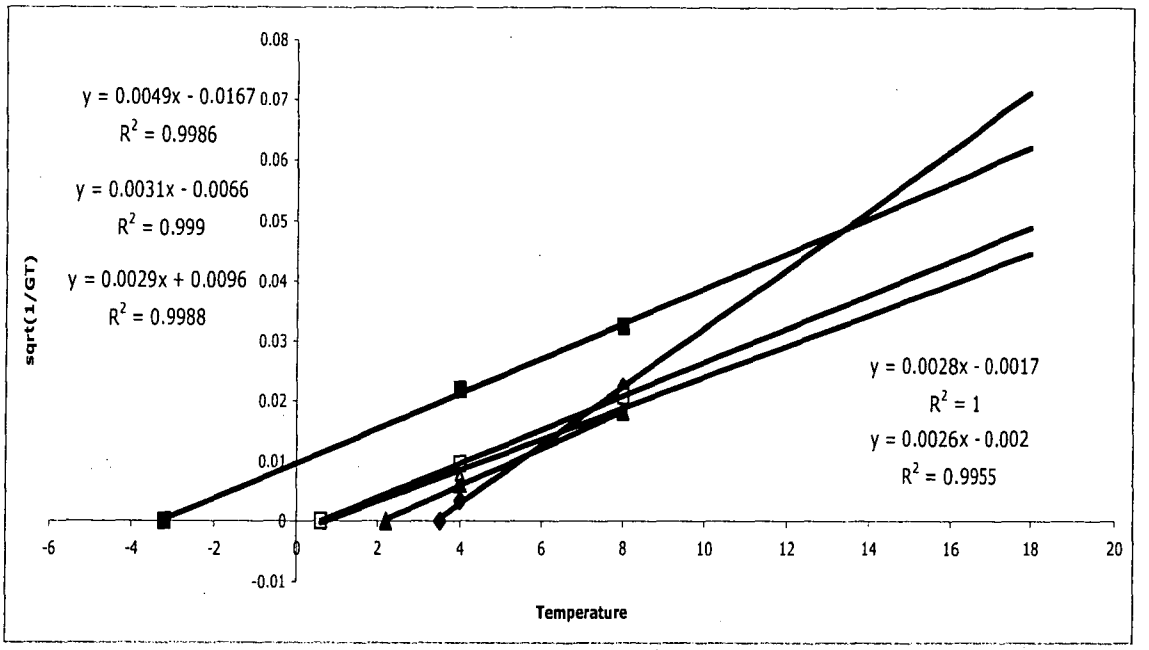

Figure 5.1.1 The pH of ham products stored at 4 °C, where (○) is the Control sample, (▒) is 10^3 CFU.mL^{-1} *L. monocytogenes* added to control, (▲) is 10^1 CFU.mL^{-1} added to control, (■) is 10^3 CFU.mL^{-1} *L. monocytogenes* and LAB added to ham and (□) is 10^1 CFU.mL^{-1} and LAB added to ham.

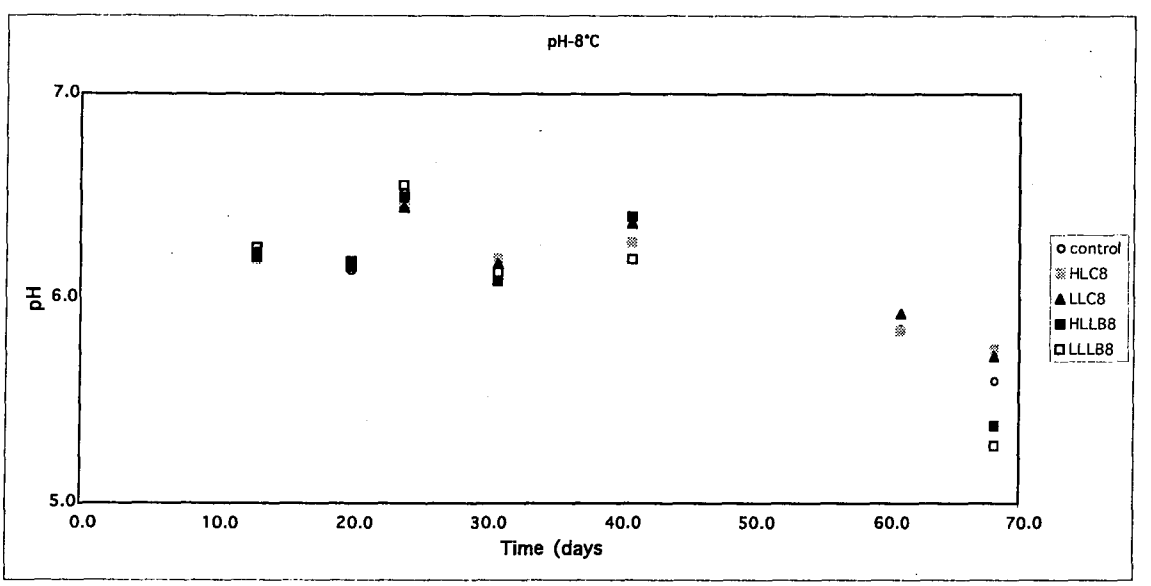

Figure 5.1.2 The pH of ham products stored at 8 °C, where (○) is the Control sample, (▒) is 10^3 CFU.mL^{-1} *L. monocytogenes* added to control, (▲) is 10^1 CFU.mL^{-1} added to control, (■) is 10^3 CFU.mL^{-1} *L. monocytogenes* and LAB added to ham and (□) is 10^1 CFU.mL^{-1} and LAB added to ham.

Table A.31 The pH values for ham products with high and low *L. monocytogenes* inoculum levels and added LAB at 4°C.

Time (days)	High *L. monocytogenes* (AV)	STD	Low *L. monocytogenes* (AV)	STD
21	6.430	0.020	6.460	0.040
38	6.450	0.017	6.297	0.146
46	6.410	0.080	6.390	0.115
60	5.910	0.036	6.007	0.071
74	5.873	0.046	5.937	0.006
81	6.260	0.087	6.243	0.025
88	6.260	0.087	6.243	0.025

Table A.32 The pH values for ham products with high and low *L. monocytogenes* inoculum levels and added LAB at 8°C.

Time (days)	High *L. monocytogenes*	STD	Low *L. monocytogenes*	STD
13	6.21	0.051	6.25	0.055
20	6.18	0.010	6.15	0.076
24	6.50	0.025	6.55	0.092
31	6.08	0.006	6.13	0.080
41	6.40	0.225	6.19	0.085
61				
68	5.38	0.169	5.28	0.061

Table A.33 The pH values for ham samples (rep 1-3) with no added LAB or *L. monocytogenes* at 4°C.

Time (days)	1 pH	2 pH	3 pH	AV	STD
21	6.46	6.410	6.45	6.440	0.026
38	6.24	6.470	6.47	6.393	0.133
46					
60					
74	6.21	6.080	6.17	6.153	0.067
81					
88	6.51	6.52	6.42	6.483	0.055

Table A.34 The pH values for ham samples with no added LAB at 4°C.

Time (days)	High *L. monocytogenes*	pH	AV	STD	pH	Low *L .monocytogenes*	pH	AV	STD
21	6.49	6.44	6.400	0.115	6.48	6.45	6.46	6.463	0.015
38	6.46	6.51	6.497	0.032	6.48	6.42	6.41	6.437	0.038
46	6.44	6.49	6.440	0.050	6.43	6.39	6.43	6.417	0.023
60	6.16	6.25	6.200	0.046	6.23	6.18	6.15	6.187	0.040
74	6.08	6.13	6.097	0.029	6.08	6.03	6.09	6.067	0.032
81									
88	6.39	6.31	6.41	0.111	6.38	6.36	6.55	6.43	0.104

Table A.35 The pH values for ham samples with no added LAB or *L. monocytogenes* at 8°C.

Time (days)	1 pH	2 pH	3 pH	AV
13	6.19	6.28	6.22	6.23
20	6.15	6.11	6.15	6.137
24	6.58	6.47	6.51	6.52
31	6.21	6.2	6.19	6.2
41				
61	5.85	5.74	5.95	5.847
68	5.42	5.49	5.87	5.593

Table A.36 The pH values for ham samples with no added LAB or *L. monocytogenes* at 8 °C.

High *L. monocytogenes*			AV	STD		Low *L. monocytogenes*		AV	STD
1	2	3			1	2	3		
6.17	6.2	6.2	6.19	0.017	6.24	6.17	6.21	6.207	0.035
6.19	6.15	6.19	6.177	0.023	6.17	6.12	6.19	6.16	0.036
6.42	6.48	6.45	6.450	0.030	6.40	6.52	6.42	6.447	0.064
6.22	6.19	6.18	6.197	0.021	6.20	6.19	6.12	6.17	0.044
6.04	6.46	6.32	6.273	0.214	6.23	6.28	6.59	6.367	0.195
5.82	5.96	5.73	5.837	0.116	5.83	5.92	6.02	5.923	0.095
5.85	5.84	5.55	5.747	0.170	5.85	5.72	5.57	5.713	0.14

Table A.37 Food Recalls due to *L. monocytogenes* contamination.

Date	Source	Volume Recalled	References
Australia			
February 2004	Primo Cocktail Frankfurts		(Recall Search Facility, 2004)
January 2004	Sliced Virginia Leg Ham Sliced Roast Beef Pickled Pork Lismore Country Meats Sliced Leg Ham		(Recall Search Facility, 2004)
December 2003	Smoked Premium Leg Ham and Silverside Eye Corn Beef		(Recall Search Facility, 2003,)
International			
December 2002	Uncle John's Pride, Kielbasa Smoked Sausages, Florida	3900 kg	FSIS (2003)
November 2002	Dakota foods Inc, Sioux Falls, cooked beef product.	959 kg	FSIS (2003)
May 2003	Illinois Firm, 5-lb. tubs of "Ble Ridge Farms made Fresh Daily Cajun Chicken Salad", packaged in individual cartons	180 kg	FSIS (2003)
May 2003	North Carolina Firm, "Cangialosi Sweet Italian sausage, beef sausage sliced, fully cooked"	81 kg	FSIS (2003)

Table A38. Composition of Welshimer's broth (WB) (Welshimer, 1963), Modified Welshimer's Broth (MWB) (minimal defined) (Premaratne *et al.*, 1991), TSB-Ye, BHI and the CDM for (Herbert and Foster, 2001).

Component	WB*	MWB*	TSB♠	BHI♠	CDM
	per L	*per* L	*per* L	*per* L	*per* L
KH_2PO_4	3.28 g	6.56 g	2.5g	-	0.85g
$Na_2HPO_4.H_2O$	15.48 g	30.96 g	-	-	0.15g
$MgSO_4.HO$	0.41 g	0.41 g	1.7g	?	0.41g
MOPS	-	-	-	-	4.18g
$FeCl_3$	-	-	-	-	0.048g
Ferric citrate	-	0.088 g	0.14g	-	-
Glucose	10.0 g	10.0 g	2.5g	2.0g	1.0g
L-Leucine	0.1 g	0.1 g	3.4g	-	0.1g
L-Isoleucine	0.1 g	0.1 g	1.5g	-	0.2g
L-Valine	0.1 g	0.1 g	2.7g	-	-
L-Methionine	0.1 g	0.1 g	0.8g	-	0.2g
L-Arginine	0.1 g	0.1 g	3.0g	-	0.2g
L-Cysteine	0.1 g	0.1 g	0.2g	-	-
L-Histidine	0.1 g	-	-	-	0.2g
L-Tryptophan	0.1 g	-	0.50g	-	-
L-Glutamine	0.6 g	0.6 g	-	-	-
Riboflavin	1.0 mg	0.5 mg	-	-	0.001g
Thiamine	1.0 mg	1.0 mg	-	-	0.005g
Biotin	0.1 mg	0.5 mg	-	-	0.001g
Thioctic acid	0.001 mg	0.005 mg	-	-	-
Tyrosine	-	-	7.2g	-	-
Threonine			1.07g		
NaCl			5.0g	5g	1.0g
Serine			0.6g		
Proline			3.4g		
Phenylalanine			1.1g		
Lysine			3.3g		0.1g
Glycine			1.4g		
Glutamic acid			9.7g		
Aspartic acid			4.3g		
Lipoic acid					10μg
Alanine			1.7g		
Nitrilotriacetic acid					0.48g
Percentage of Total amino acids	0.14%	0.12%	1.97%		0.1%

♠ Bridson, 1998.

* (Premaratne *et al.*, 1991)